Mn^{2+} tolerance in barley (*Hordeum vulgare* L.) and its contribution to waterlogging tolerance

by

Xin HUANG

B.Sc.(Agr.)

South China Agricultural University, China

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Declaration

Declaration of Originality

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Xin HUANG

University of Tasmania

September 2017

Statement of Co-authorship

This thesis was completed during my PhD study in the School of Land and Food at University of Tasmania. This thesis contains no experimental results that have previously been presented for any degree at this or other institution.

The following people and institutions contributed to the publication of work undertaken as part of this thesis:

Xin Huang, School of Land and Food, University of Tasmania

Meixue Zhou, School of Land and Food, University of Tasmania

Sergey Shabala, School of Land and Food, University of Tasmania

Lana Shabala, School of Land and Food, University of Tasmania

Zed Rengel, School of Agriculture and Environment, The University of Western Australia

Yun Fan, School of Land and Food, University of Tasmania

Natsuko I Kobayashi, School of Agricultural and Life Science, The University of Tokyo

Xiaojian Wu, College of Agriculture and Biotechnology, Zhejiang University

GuoPing Zhang, College of Agriculture and Biotechnology, Zhejiang University

Author details and their roles:

Paper 1: Zhang X, **Huang X**, Zhou M, Shabala L, Koutoulis A, Shabala S, (2015) Plant breeding for flood tolerance: advances and limitations. *In*: P.K. Jaiwal, R.P. Singh & O.P. Dhankher (eds.) *Genetic Manipulation in Plants for Mitigation of Climate Change.* Springer, India. – Located in *Chapter II*, 2.1.3 and 2.2

Author contribution: X.Z., X.H., M.Z., L.S., A.K., and S.S. participated in manuscript preparation equally; S.S. reviewed and revised the manuscript.

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Author contribution: S.S., M.Z., L.S., Z.R. and X.H. conceived and designed the research; X.H. and X.W. conducted experiments; X.H. analysed data and prepared the manuscript; S.S., M.Z., Z.R., L.S. and G.Z. reviewed and revised the manuscript

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Author contribution: M.Z., Z.R. and X.H. conceived and designed the research, Y.F. and X.H. conducted the experiment, data analysis. and manuscript preparation; M.Z., S.S., Z.R. and L.S. reviewed and revised the manuscript.

We the undersigned agree with the above stated "proportion of work undertaken" for each of the above published (or submitted) peer-reviewed manuscripts contributing to this thesis:

Signature: _____ _____

Meixue ZHOU	*Holger MEINKE*
Supervisor	*Head of School*
School of Land and Food	*School of Land and Food*
University of Tasmania	*University of Tasmania*

Date: 06.Sep.2017

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List of Abbreviations

BSM	basic-salt media solution
CAX	Ca^{2+}/H^{+} exchanger
CDF	cation diffusion facilitator
CDS	coding DNA sequence
DACC	depolarisation-activated calcium channel
DH	double haploid
DMA	2'-deoxymugineic acid;
DPI	diphenyleneiodonium
DW	dry weight
EDTA	ethylenediaminetetraacetic acid
FRD	ferric chelate reductase defective transporter
FRO	ferric reductase oxidase
HACC	hyperpolarization-activated Ca^{2+} channels
HAK	high-affinity K^{+} transporter
IM	interval mapping
IRT	iron-regulated transporter
LIX	liquid ionic exchanger
LOD	logarithm of the odds
MES	2-(*N*-morpholino)ethanesulfonic acid
MIFE	microelectrode ion flux estimation
MP	membrane potential
MQM	multiple QTL model
MTP	metal tolerance protein
MZ	mature zone
NA	nicotianamine
NADPH	nicotinamide adenine dinucleotide phosphate
NRAMP	natural resistance-associated macrophage protein
NSCC	non-selective cation channel
PCD	programmed cell death
PCR	polymerase chain reaction
PM	plasma membrane
POD	peroxidase
QTL	quantitative trait loci
ROS	reactive oxygen species
RT-qPCR	real time quantification polymerase chain reaction
SOD	superoxide dismutase
TEA	tetraethylammonium chloride
TG	thapsigargin
WL	waterlogging
YSL	yellow stripe-like transporter

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Abstract

Vast agricultural areas are affected by flooding causing up to 80% yield reduction and resulting in multibillion dollar losses. In recent years, the occurrence of flooding has increased though human activities, especially in agricultural systems with poor drainage. Therefore, waterlogging stress is becoming one of the main challenge of the modern agriculture. Of all the cereals, barley (*Hordeum vulgare* L.) is a widely adaptable crop, which is ranked fourth among grains in quantity produced behind maize (*Zea mays* L.), rice (*Oryza sativa* L,) and wheat (*Triticum aestivum* L,). The less complex barley genome also makes it a useful species to understand physiological and molecular aspects of plant adaptation to the flooding stress and to improve their adaptive abilities to confront environmental constrains.

Waterlogging tolerance is a complex trait affected by various factors including soil characteristics, temperature, plant developmental stage, microbe activities and oxygen availability. Understanding the mechanisms of waterlogging tolerance make it possible for plant breeders to target individual physiological traits and create barley breeding materials with enhanced waterlogging tolerance. Up to now, the focus of plant breeders was predominantly on alleviating detrimental effects of anoxia, while other (potentially equally important) traits were essentially neglected. One of these is the soil elemental toxicity. Excess water triggers a progressive decrease in the soil redox potential, thus increasing the concentrations of Mn^{2+} and Fe^{2+} that can be toxic to plants, when exceeding a threshold concentration. Cellular detoxification and exclusion are the main strategies for plants to resist excess ion concentration in soil. However, specific details of their coordination and the relative contribution of these components towards manganese toxicity tolerance in barley have not been fully revealed. Besides, the linkage between ion toxicity tolerance and waterlogging stress tolerance is still poorly understood, although tolerance to one or more elemental toxicities can be an essential trait to improve plant performance in waterlogged soils.

Accordingly, the major aim of this PhD project was to investigate the physiological and molecular aspects of manganese toxicity tolerance associated with waterlogging stress tolerance. The following specific objectives were addressed:

To quantify the relative contribution of Mn^{2+} toxicity to waterlogging stress tolerance;

To develop a rapid screening method and screen a large number of barley varieties;

To identify QTLs controlling tolerance to manganese toxicity in barley associated with tolerance of waterlogging stress;

To investigate physiological and molecular mechanisms conferring manganese tolerance.

Working along these lines, a broad range of barley (*Hordeum vulgare* and *Hordeum spontaneum* L.) genotypes contrasting in waterlogging stress tolerance were used to investigate its linkage with manganese toxicity tolerance. In total, twenty barley genotypes (including three wild barleys) contrasting in waterlogging stress tolerance were studied for their ability to cope with the toxic (1 mM) amounts of Mn^{2+} in the root rhizosphere. Under Mn^{2+} toxicity, chlorophyll content of most waterlogging-tolerant genotypes (TX9425, Yerong, CPI-71284-48 and CM72) remained above 60% of the control value, whereas sensitive genotypes (Franklin and Naso Nijo) had a chlorophyll content less than 35% of the control. Manganese concentration in leaves was not related to visual Mn^{2+} toxicity symptoms, suggesting that various Mn^{2+} tolerance mechanisms might have operated in different tolerant genotypes, *i.e*. avoidance *versus* tissue tolerance. The overall significant ($r = 0.60$) correlation between tolerances to Mn^{2+} toxicity and waterlogging in barley suggests that plant breeding for tolerance to waterlogging traits may be advanced by targeting mechanisms conferring tolerance to Mn^{2+} toxicity, at least in this species.

Direct selection (using only agronomic traits) for stress tolerance is easily affected by environments thus less effective. Marker assisted selection (MAS) could provide an indirect selection process which can effectively reveal distinct genetic differences but not merely on trait itself. Therefore, further studies were conducted in specific doubled-haploid populations to determine whether the same genes are responsible for Mn^{2+} and waterlogging tolerance. A total of 177 lines from Yerong/Franklin population were used to identify QTL conferring Mn^{2+} tolerance and another 188 DH lines from TX9425/Naso Nijo were used to validate

the QTL identified in the Yerong/Franklin population. Seven QTLs were identified from these two populations. Among all, four QTL controlling plant survival under manganese toxicity determined almost 40% of phenotypic variation. Two significant QTL for leaf chlorosis were identified at a similar position as those for plant survival on chromosome 3H and 6H, explaining 22.1% and 7.5% of phenotypic variation respectively. In the TX9425/Naso Nijo DH population, only one significant QTL accounting for plant survival was identified which was located at a same position on chromosome 3H as the major QTL identified in the Yerong/Franklin DH population. Based on the major QTL on chromosome 3H, three candidate genes (*POD*, *KAT3*, *HMA*) for this QTL were identified, suggesting antioxidant system and potassium transport might play a substantial role in coping with manganese toxicity.

We then used the MIFE (microelectrode ion flux measurement) technique to study some aspects of manganese stress signalling, focusing on K^+ transport (as per about QTL findings). K^+ retention plays a pivotal role in conferring many abiotic stress tolerances in plants. In this work, ten selected barley genotypes were used to study Mn-induced changes in K^+ transport. These fluxes were then related to appropriate changes in fluxes of Ca^{2+} (a known second messenger) and H^+ (a proxy for H^+-ATPase activity). All genotypes responded to Mn treatment by net K^+ influx, while net Ca^{2+} and H^+ efflux was observed after adding 1 mM Mn^{2+}. No significant difference among genotypes was found. Several inhibitors were used to understand the specific signal pathway affected by manganese. Manganese-induced K^+ uptake and Ca^{2+} efflux were significantly inhibited by TEA (a blocker of K^+ channels) and vanadate (H^+-ATPase inhibitor). However, a significant K^+ and Ca^{2+} leakage was measured in DPI-pretreated root when applied Mn treatment, suggesting that NADPH-oxidase may play an essential role in regulating Mn uptake. High manganese concentration did not significantly affect net Ca^{2+} flux and net K^+ flux in Gd^{3+}, La^{3+} or TG (thapsigargin, endomembrane Ca^{2+} channel inhibitor) pretreated roots. The above results suggest that both non-selective cation channels and Ca^{2+}/H^+ exchangers contribute to manganese uptake and transport in barley roots. Hypoxic conditions also trigger a burst in reactive oxygen species (ROS), resulting in a significant K^+ efflux. This work has shown that hypoxia enhanced sensitivity to exogenous H_2O_2 while additional Mn ion efficiently alleviated the

impact of hypoxia on intracellular K^+ homeostasis. The reported up-regulated expression of *HAK* gene also suggests that manganese may play an important role by signalling K^+ deficiency and enabling plants with mechanisms for better K^+ retention to confront stress.

In conclusion, this project has found that different barley genotypes adopt different strategies to resist manganese toxicity. Both exclusion and internal tolerance mechanisms contribute to Mn^{2+} tolerance. Tolerance to Mn^{2+} showed a significant positive correlation with waterlogging tolerance. However, Mn^{2+} does not appear to be toxic in roots. The ability of roots to retain K^+ was proven to be one of the key traits conferring tolerance to numerous stress. Mn was able to trigger a hyperpolarisation of the plasma membrane, leading to significant K^+ uptake. NADPH oxidase-mediated apoplastic H_2O_2 production may be causally related to ROS inducible Ca^{2+} uptake systems contributing to Mn uptake. Also, *HvHAK5* transporter was found to be involved in maintenance of K^+ content in root, which was identified at the major QTL on chromosome 3H associated with manganese toxicity. This QTL could therefore be used in breeding programs to enhance both manganese toxicity tolerance and waterlogging tolerance.

Chapter I: General introduction

1.1 Waterlogging stress and agricultural crop production

Waterlogging is one of the most severe constraints affecting agricultural crop production (Setter and Waters 2003; Shabala 2011), extending from sandy duplex soil characterised by intermittent waterlogging to heavy clay vertisols that can be waterlogged for prolonged periods (Setter and Waters 2003). Depletion of oxygen in the soils environment is the main detrimental consequence caused by waterlogging. The reduced water and nutrient absorption by roots generally occurs under either partial (hypoxia) or complete (anoxia) depletion of oxygen (Malik *et al*. 2002). During waterlogging or submergence, plants are exposed to a decrease in the oxygen supply due to the slow diffusion rate of oxygen in water and its limited solubility (Armstrong, 1978). Plant growth is greatly inhibited in hypoxic or anoxic soils, resulting in significant yield reduction (Steffens *et al*. 2005; Dickin and Wright 2008).

In Australia, transient waterlogging occurs primarily in sandy duplex soils, where rainfall rapidly penetrates a sandy topsoil and accumulates above a compacted clay subsoil with low hydraulic conductivity at 5 -> 100 cm depth (Tennant *et al*. 1992). More than 0.5 million cereal crops are subjected to waterlogging during August in Western Australia (Khabaz-Saberi *et al*. 2006). In the USA, waterlogging affects around 16% of soils, and causes large crop loss due to excess water (excluding flooding), which is second only to drought (Zhou 2011). Early estimates indicate that waterlogging affects 10 – 15 million ha of wheat each year (Sayre *et al*. 1994). In barley, waterlogging can reduce yields on average by 20 to 25%, and the loss may increase beyond 50% depending on the stage of plant development (Setter *et al*. 1999). In recent years, the probability of flooding is gradually raised by human activities such as removal of natural vegetation, improvement of drainage systems further up the catchment, overgrazing by cattle, and straightening of meanders to facilitate shipping (Blom and Voesenek 1996). As alarmingly changing earths' average temperature, the erratic rainfall, rise in sea level caused by increasing melting glaciers and shift in the native climate-spectrum will exaggerate the problem on agricultural cops in the near future (Griggs and Noguer 2002; Irfan *et al*. 2010).

Barley (*Hordeum vulgare* L.) is a major cereal grain crops, with an annual production of around 150 million tonnes during past decade, that is only exceeded by rice (*Oryza sativa* L,), wheat (*Triticum aestivum* L,) and maize (*Zea mays* L.) (Statista 2017). Among other cereals, barley is the more susceptible to waterlogging stress (Setter and Waters 2003). Improving tolerance to waterlogging stress is one of the major objectives in barley breeding program in China and Japan (Zhou *et al.* 2007). Australia produces more than 40% of the world's malting barley and 20% of the feed barley (AEGIC 2015). However, most Australian commercial barley varieties are waterlogging sensitive, resulting in economic losses of millions of dollars. Due to the complexity of adaptation to waterlogging stress, current selection methods to screen this trait are imprecise and inefficient (Bertholdsson 2013), which makes selection of resistance to waterlogging difficult. Importantly, breeding for waterlogging tolerance in barley has been focused mainly on oxygen-related traits. At the same time, oxygen deficiency is not the only constraint reducing plant growth in the flooded soils.

1.2 Waterlogging stress and elemental toxicity

Following flooding, excess water and associated reduction in oxygem availability in the soil results in a progressive decrease in the soil redox potential (Eh). Waterlogging causes reduction of oxidized ions including Fe^{3+} and Mn^{4+}, enhancing the concentration of Fe^{2+} and Mn^{2+} over the plant nutritional requirements at any plant development stage (Khabaz-Saberi *et al.* 2006; Bailey-Serres and Voesenek 2008; Khabaz-Saberi and Rengel 2010). High concentrations of Fe and Mn in waterlogging affected acid soils are reported as crucial restrictions for intolerant wheat genotypes, leading to 2- to 10-fold increase in shoot Mn and Fe concentrations (Khabaz-Saberi *et al*. 2006) and resulting in 25% to 50% decrease in shoot dry weight (Khabaz-Saberi and Rengel, 2010). Wagatsuma et al. (1990) also reported that Mn toxicity may be involeved in some specific cases where waterlogging occurs.

Various agronomic investigation has been aimed at minimising the adverse effects of waterlogging on crop growth and development (Samad *et al*. 2001). Plant breeding for waterlogging tolerance has traditionally focused on traits related to

improve oxygen availability, such as preventing oxygen loss from non-meristematic root tissues or improving oxygen transport to, or storage in, the root (Jackson and Armstrong 1999), as well as formation of aerenchyma in roots (Mano *et al*. 2007). However, tolerance to Mn^{2+} of Fe^{2+} toxicity has never been directly targeted in any breeding programmes aimed at improving waterlogging tolerance in barley. Meanwhile, soil elemental toxicity may be one of the key factor in diverse environment, affecting plant waterlogging tolerance, and tolerance to one or more elemental toxicities can be substantial that to improve the overall plant performance in waterlogged soil (Khabaz-Saberi *et al*. 2006). In wheat, tolerance to multiple ion-toxicities as an innovative approach was reported to improve yields conferring better root and shoot growth in waterlogging – affected acid soil (Khabaz-Saberi and Rengel, 2010).

1.3 Objectives and research aims

The major aim of this project was to investigate the physiological and molecular aspects of manganese toxicity tolerance associated with waterlogging stress tolerance. The following specific objectives were addressed:

To develop an efficient screening methodology for manganese toxicity in barley breeding programs.

The availability of efficient screening tools is crucially important to select potential genotypes with promising tolerance of manganese toxicity in large populations in a barley breeding program. A technique that can give a rapid, reliable, quantitative assessment of the impacts caused by the stress factor is required. Two barley genotypes contrasting in waterlogging tolerance were used for this study.

To quantify the relative contribution of Mn^{2+} toxicity to waterlogging stress tolerance.

In this part, the aim was to investigate the relationship between Mn^{2+} toxicity and the overall waterlogging stress tolerance in barley. This was achieved by physiological assessment of twenty barley genotypes and correlating the overall waterlogging stress tolerance in this species with changes in plant agronomical and physiological characteristics and manganese content in shoot.

To identify QTLs controlling tolerance to manganese toxicity in barley associated with tolerance of waterlogging tolerance.

As most of the previous studies have been targeting manganese use efficiency (Pallotta *et al.* 2000; McDonald *et al.* 2001; Hebbern *et al.* 2005), no QTL have been reported for the tolerance to manganese toxicity in barley. In this part, we used two selected DH populations to identify QTL controlling tolerance to manganese toxicity. Chlorophyll content and plant survival under Mn^{2+} toxicity were used as physiological markers for Mn^{2+} tolerance.

To investigate physiological and molecular mechanisms conferring manganese tolerance.

The main objective of this part was to answer several aspects of manganese tolerance associated with waterlogging tolerance using the MIFE technique for non-invasive microelectrode ion flux measurements. The overall goal of this work was to provide a understanding of the signal transduction pathways affected by manganese ion.

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Chapter II: Literature review

2.1 Detrimental effects of waterlogging stress

2.1.1 Oxygen availability in flooding soils

Excess water results in complex changes in several environmental parameters, leading to hypoxia and progressively to anoxia. Therefore, oxygen depletion in the soil environment is the main detrimental consequence caused by waterlogging. As water saturation in soil increases, air spaces are filled, leading to the modification of several soil physico-chemical characteristics (Kirk *et al*. 2003; Dat *et al*. 2004). The depletion of O_2 over time is considered mainly as the consequence of the soil by microbial respiration (Grunth *et al*. 2008). Water dissolves approximately at 230 mmol / m^3 under normal conditions, however, oxygen level falls below 50 mmol / m^3 as a result of hypoxia (Grichko and Glick 2001). During waterlogging or submergence, plants are exposed to a decrease in the oxygen supply due to the slow diffusion rate of oxygen in water and its limited solubility (Armstrong 1978). Within a few hours to several days, the slow diffusion rate, low solubility, and increased microbial mechanisms not only reduce soil oxygen level, but also induce generation of toxic organic and inorganic compounds in the soil (Gambrell and Patrick 1978; Ponnamperuma 1984; Gambrell *et al*. 1991). Roots are directly injured by deprivation of oxygen in waterlogged soil, resulting a rapid death of seminal roots (Malik *et al*. 2001).

2.1.2 Metabolic perturbations and energy crisis

Upon waterlogging, oxygen diffusion in water is 10^4 times slower than the oxygen diffusion in the air, resulting in hypoxia (oxygen deficiency) or anoxia (oxygen exhaustion) conditions around roots (Bailey-Serres and Voesenek 2008). Oxygen is the final electron acceptor in the mitochondrial electron transport chain. During waterlogging, oxygen deprivation is the main factor inhibiting plant growth, which develops rapidly in the dark and in non-photosynthetic cells. Consequently, the reduced availability of oxygen induces a rapid ATP reduction in such hypoxia conditions, and decreased energy availability occurs (Gibbs and Greenway 2003; Colmer and Voesenek 2009). This decreased energy availability impacts the metabolic balance in plant tissues, mainly on account of reduced NADH oxidation

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(Bailey-Serres and Voesenek 2008; Voesenek and Sasidharan 2013). Cells cope with this constraint by relying activities of many glycolytic and fermentative enzymes, which increase in adventitious roots to generate ATP and regenerate NAD^+ (Bailey-Serres *et al*. 2012). However, energy generation through glycolysis or fermentation under waterlogging conditions is significantly less than that from mitochondrial respiration. Resulted from this energy deficiency, nitrogen uptake and its transportation are impeded, leading to leaves chlorosis and ultimately in death (Trought and Drew 1980). In addition, energy-dependent ion transport mechanisms can be inhibited during root hypoxia (Shabala *et al*. 2014), which is a critical indicator to evaluate roots and specific cell types for their Na^+, Cl^+ and K^+ homeostasis during combined hypoxia and salinity. (Kotula *et al*. 2015).

Waterlogging also induces cellular overproduction of reactive oxygen species (ROS) including superoxide radical ($O_2^{\cdot}$), hydroxyl radical ($OH^{\cdot}$), hydroperoxyl radical ($HO_2^{\cdot}$), and hydrogen peroxide (H_2O_2). These ROS can not only be harmful to cellular metabolism and may induce the oxidative stress (Overmyer *et al*. 2003), but also be used as signalling molecules which involve in tolerance mechanisms (Wong *et al*. 2004; Gechev *et al*. 2006). Meanwhile, antioxidative enzymes (including POD, SOD, CAT) and non-enzymatic compounds that ameliorate ROS are effective at conferring waterlogging tolerance (Apel and Hirt 2004; He *et al*. 2012).

2.1.3 Changes in soil redox potential and elemental toxicity#

2.3 Regulation of ionic homeostasis in plants affected by waterlogging

2.3.1 H^+pumping and cytosolic pH regulation

H^+ pumps are major consumers of ATP involved in cytosolic pH regulation, and are directly responsible for the maintenance of membrane potential at the root plasma membrane (Greenway and Gibbs 2003; Felle 2005; Koizumi *et al*. 2011; Teakle *et al*. 2013). However, reduced energy availability (mentioned in 2.1.2) makes its operation questionable. The low energy status under oxygen deficient condition induces a substantial depolarisation of membrane potential, typically 40 to 80 mV less negative than the initial (steady-state) value (Zeng *et al.* 2014). This significantly disturbs transport processes for most essential cations (such as K^+ and NH_4^+) through voltage-gated uptake channel. It also has detrimental consequences for H^+ symport-mediated uptake of anions such as NO_3^- and SO_4^{2-} (Wang *et al*. 2013). Another type of H^+-ATPase is the tonoplast-located V-ATPase which has at least 11 different subunits (Dietz *et al*. 2001). It plays a critical role in preventing the cytosolic acidification in vacuole under anoxic conditions (Greenway and Gibbs 2003; Felle 2005; Koizumi *et al*. 2011). In addition, the vacuolar H^+-pyrophyophatase pump (termed V-PPase) existing at the tonoplast membranes (Dietz *et al*. 2001) is often considered as an alternative mechanism switched from V-ATPase-driven H^+ transport that beneficial to anoxically-treated roots (Gibbs and Greenway 2003). An evidence also shown that rice seedlings possibly maintain tonoplast energisation under anoxia by increased transcript level of V-PPase (Carystinos *et al*. 1995), suggesting V-PPase is an essential survival strategy for plant under anoxia. Therefore, creating transgenic crops with overexpressed PPase activity may be a potential approach in developing waterlogging-tolerant crops (Koizumi *et al*. 2011; Shabala *et al*. 2014).

2.3.2 K^+ retention under stress conditions

Potassium comprises up to 10% of plant dry matter (Britto and Kronzucker 2008) and plays an important role in plants and is involved in multiple cellular processes, including osmoregulation, leaf and stomata movements, enzyme activation, control of membrane polarization xylem loading, charge balancing, cytoplasmic pH maintenance, stabilisation of protein synthesis, and energy conservation across membranes (Dreyer and Uozumi 2011; Anschütz *et al*. 2014; Shabala and Pottosin

2014). As most of these processes are involved in plant adaptation to environment constraints, K^+ retention plays a pivotal role in conferring many abiotic stress tolerances in plants, such as salinity (Chen *et al.* 2005; Cuin *et al*. 2005), hypoxia (Zeng *et al.* 2013), and drought (Hu and Schmidhalter 2005).

Under waterlogging condition, root K^+ uptake is notably reduced upon oxygen depletion (Elzenga and van Veen, 2010). In barley roots, a significant K^+ efflux was observed under hypoxia condition (Ma *et al.* 2016), and decreased K^+ content up to 40% (Zeng *et al*. 2013). The ability of roots to maintain a better cytosolic K^+ homeostasis and K^+ channel activity was regarded as an essential component for plants to acclimate to hypoxia (Mugnai *et al*. 2011; Barrett-Lennard and Shabala 2013). It was also suggested that K^+ loss is critical to initiate plant programmed cell death (PCD) (Peters and Chin 2017). High cytosolic K^+ levels are essential to suppress activity of caspase-like proteases and endonucleases in plants, and decrease in the cytosolic K^+ pool may result in activation of these catabolic enzymes triggering PCD (Shabala *et al*. 2007; Demidchik *et al*. 2010). However, in some tissues, this process may be of adaptive significance, and it was argued that such a PCD eliminated cells in root cortex and generated large gas spaces, contributing to formation of aerenchyma (Shabala *et al.* 2014). The gaseous hormone ethylene promotes the formation of aerenchyma by accumulation in plant organs during waterlogging or submergence (Visser and Voesenek 2005; Steffens and Sauter 2009).

2.3.3 Ca^{2+} homeostasis and signalling

Anoxia induces a rapid increase in the cytosolic free Ca^{2+} concentration in plant cell (Yemelyanov *et al*. 2011), which is believed to be critical for gene expression in acclimation to the change at the cellular level, acting as a key transducer of changes in oxygen availability (Subbaiah and Sachs 2003). The utilisation of several inhibitors which block Ca^{2+} uptake has also given evidence to the linkage between Ca^{2+} accumulation and plant survival under anoxia (Sedbrook *et al*. 1996; Yemelyanov *et al*. 2011). The availability of free cytoplasmic calcium regulates the formation of aerenchyma as influx of Ca^{2+} into the cytoplasm is a necessary step in the process leading to cell death (He *et al*. 1996; Visser and Voesenek 2005;

Fagerstedt 2010), and this formation can be impeded by inhibitors of free Ca^{2+} released in roots (He *et al*. 1996).

Ca^{2+} signalling can also trigger activation of NADPH oxidase, resulting in an increase of the production of H_2O_2. H_2O_2 further activates hyperpolarization-activated Ca^{2+} channels, resulting in the Ca^{2+} influx, which also initiates this cycle (Laohavisit and Davies 2007). In *Arabidopsis*, exogenous H_2O_2 application leads to the increase of Ca^{2+} influx in roots (Demidchik et al. 2007), and a similar result also can be observed in barley (see *Chapter V*). The increase of H_2O_2 and free Ca^{2+} in the hypoxia stress in roots leads to ethanolic fermentation, suggesting another substantial strategy for plants to be tolerant under waterlogging stress (Baxter-Burrell *et al*. 2002).

2.4 Crop breeding for tolerance of waterlogging stress and manganese toxicity

Due to the complexity of plant waterlogging tolerance and the multigenic nature of the trait, the phenotypic features associated directly with waterlogging tolerance remain unclear and controversial (Pezeshki *et al.* 1999; Smethurst *et al.* 2005; Teakle *et al.* 2010). As the importance of oxygen in plant metabolism in waterlogging soils, environmental oxygen availability or plant ability to retain oxygen in root is given priority in breeding program, while soil elemental toxicity is essentially neglected, even the importance of this toxicity was elucidated on many occasions (Khabaz-Saberi *et al.* 2006; Shabala 2011). A clear understanding of the genetic background and mechanisms to improve waterlogging tolerance is critical in both research and breeding program. During past decades, many QTL have been reported for tolerance of waterlogging or submergence based on various agronomical and morphological characteristics in different doubled-haploid (DH) populations (Qiu *et al*. 2007; Li *et al.* 2008; Zhou 2011; Zhang *et al*. 2013; Zhang *et al.* 2016). However, no QTL has been reported for the tolerance to soil elemental toxicity so far, even several transporters mentioned above have been reported to be responsible for accumulation and sequestration (Pittman 2005; Pedas *et al.* 2008; Cailliatte *et al.* 2010; Sasaki *et al.* 2012; Chen *et al.* 2013).

2.5 Conclusion

The major focus of research on waterlogging tolerance mechanisms has been on understanding the ability of plants to deal with the lack (or a complete absence) of oxygen. Despite some significant breakthroughs such as understanding the role of ethylene in formation of aerenchyma and introduction of *SUB* genes in development of submergence-tolerant cultivars (Septiningsih *et al.* 2009; Fukao *et al.* 2012; Septiningsih *et al.* 2015), the progress in the field is much slower, and most of crops suffer from substantial losses under waterlogged conditions. One of the reason for this is that under field conditions plants are exposed to multiple constraints, not only oxygen deficiency. Thus, our limited understanding of coordination and linkage between waterlogging stress and elemental toxicity delayed the efforts to breed cereals in superior overall waterlogging tolerance. Tolerance to multiple ion toxicity can be potentially utilised as an innovative approach in breeding programme in great extent. However, more work is required to better understand the role of elemental toxicity tolerance mechanisms, to implement specific aspects (physiological or molecular level) conferring plant waterlogging stress tolerance traits.

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Chapter III: Linking waterlogging tolerance with Mn^{2+} toxicity: a case study for barley[#]

Xin Huang[1], Sergey Shabala[1], Lana Shabala[1], Zed Rengel[2], Xiaojian Wu[3], Guoping Zhang[3] and Meixue Zhou[1]

[1]School of Land and Food, University of Tasmania, Hobart, TAS 7001, Australia

[2]School of Agriculture and Environment, The University of Western Australia, Perth, WA 6009, Australia

[3]College of Agriculture and Biotechnology, Zhejiang University, Hangzhou, China

Abstract

Vast agricultural areas are affected by flooding causing up to 80% yield reduction and resulting in multibillion dollar losses. Up to now, the focus of plant breeders was predominantly on detrimental effects of anoxia, while other (potentially equally important) traits were essentially neglected; One of these is the soil elemental toxicity. Excess water triggers a progressive decrease in soil redox potential, thus increasing the concentration of Mn^{2+} that can be toxic to plants if above a specific threshold. This work aimed to quantify the relative contribution of Mn^{2+} toxicity to waterlogging stress tolerance, using barley as a case study. Twenty barley (*Hordeum vulgare*) genotypes contrasting in waterlogging stress tolerance were studied for their ability to cope with the toxic (1 mM) amounts of Mn^{2+} in the root rhizosphere. Under Mn^{2+} toxicity, chlorophyll content of most waterlogging-tolerant genotypes (TX9425, Yerong, CPI-71284-48 and CM72) remained above 60% of the control value, whereas sensitive genotypes (Franklin and Naso Nijo) had their chlorophyll content less than 35% of the control. Manganese concentration in leaves was not related to visual Mn^{2+} toxicity symptoms, suggesting that various Mn^{2+} tolerance mechanisms might have operated in different tolerant genotypes, *i.e*. avoidance *versus* tissue tolerance. The overall significant ($r = 0.60$) correlation between tolerances to Mn^{2+} toxicity and waterlogging in barley suggests that plant breeding for tolerance to waterlogging traits may be advanced by targeting mechanisms conferring tolerance to Mn^{2+} toxicity, at least in this species.

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Keywords

breeding, chlorophyll, *Hordeum* vulgare, Mn^{2+} toxicity, sequestration, tissue tolerance, waterlogging tolerance

3.1 Introduction

Waterlogging is one of the most severe stresses affecting crop production worldwide, particularly in (i) duplex soil (sand over clay) characterised by intermittent waterlogging and (ii) heavy clay vertisols that can remain waterlogged for long periods of time (Setter and Waters 2003; Shabala 2011). In barley, waterlogging can reduce yields by 20 - 25%, and the loss may increase beyond 50%, depending on the stage of plant development (Setter *et al.* 1999). Waterlogging can also hamper wheat plant development, particularly in sensitive genotypes (Khabaz-Saberi and Rengel 2010). Due to the complexity of waterlogging, which is easily affected by the environment, it is not effective to make direct selection for waterlogging tolerance in segregating populations. Understanding the mechanisms of waterlogging tolerance makes it possible for plant breeders to target individual physiological traits and pyramid different tolerance-related traits to create barley pre-breeding materials with enhanced waterlogging tolerance.

Waterlogging is a complex constraint, altering plant metabolism in a multitude of ways. In addition to reducing oxygen availability and causing hypoxia (Bailey-Serres and Voesenek 2008; Colmer and Voesenek 2009; Voesenek *et al.* 2013), excess water triggers a progressive decrease in soil redox potential (>300 mV in the first 2 weeks; Zeng *et al.* 2013). Many metal oxides, including Fe^{3+} and Mn^{4+}, are utilised as alternative electron acceptors, thus increasing the concentration of Fe^{2+} and Mn^{2+} that are toxic to plants above specific thresholds (Pezeshki and DeLaune 1998; Bailey-Serres and Voesenek 2008; Igamberdiev and Hill 2009; Khabaz-Saberi and Rengel 2010).

A significant increase in Fe^{2+} and Mn^{2+} concentrations (20- and 80-fold, respectively) has been reported in maize leaves while growing in sandy loam soil subjected to 34 days of flooding (Ashraf and Rehman 1999). Unlike Al, excess Mn^{2+} generally affects shoots more than roots (Foy *et al.* 1978). Although the

expression of Mn^{2+} toxicity varies considerably among plant species, the typical symptom of Mn^{2+} toxicity for many plants is marginal leaf chlorosis and necrosis, and appearance of brown necrotic spots on older leaves (Foy *et al.* 1978; El-Jaoual and Cox 1998). Symptoms of Mn^{2+} toxicity usually appear first on the old leaves and extend to the younger leaves as the plant grows (Wang *et al.* 2002).

Plant breeding for waterlogging tolerance has traditionally targeted traits related to increased oxygen availability, such as preventing oxygen loss from non-meristematic root tissues or improving oxygen transport to, or storage in, the root (Jackson and Armstrong 1999). Even though the importance of toxicity of ions and secondary metabolites as components of waterlogging stress has been emphasised on many occasions (Khabaz-Saberi *et al.* 2006; Shabala 2011), the concept of improving waterlogging tolerance by targeting tolerance to ion toxicities has yet to be fully accepted by the breeding community. Nevertheless, genotypes with superior ability to reduce detrimental effects of secondary metabolites and toxic ions such as Mn^{2+} were shown to perform better in waterlogged soil (Pang *et al.* 2007; Khabaz-Saberi and Rengel 2010; Khabaz-Saberi *et al.* 2012). Tolerance to one or more ion toxicities can be an essential trait to improve plant performance in waterlogged soils (Khabaz-Saberi *et al.* 2006). Multiple ion toxicity tolerance as an innovative approach was reported to improve wheat root and shoot growth in waterlogging-affected acid soils (Khabaz-Saberi and Rengel 2010). Variation in Mn^{2+} tolerance was also found in Australian wheat (Scott *et al.* 1998; Khabaz-Saberi *et al.* 2010) and rapeseed germplasm (Moroni *et al.* 2003). However, tolerance to Mn^{2+} toxicity has never been targeted in breeding programmes to improve waterlogging tolerance in barley, and there is no reliable protocol for screening barley germplasm for Mn^{2+} tolerance.

In the present work, we used screening methods based on hydroponics to investigate the causal relationship between Mn^{2+} toxicity and waterlogging stress tolerance in barley. A strong and significant ($r = 0.60$) correlation between these two traits observed while screening 20 barley varieties of barley suggests that plant breeding for the latter trait may be advanced by targeting mechanisms conferring tolerance to Mn^{2+} toxicity. Importantly, Mn^{2+} concentration in leaves was not related to visual Mn^{2+} toxicity symptoms, suggesting that various Mn^{2+} tolerance mechanisms might operate in different tolerant genotypes.

3.2 Materials and methods

3.2.1 Plant material and growth conditions

Two barley (*Hordeum vulgare*) genotypes, Naso Nijo (waterlogging sensitive) and TX9425 (waterlogging tolerant) were used to optimise conditions for screening plant tolerance to Mn^{2+} toxicity. Eighteen other barley genotypes (CM72, CPI-71284-48, CXHKSL, Franklin, Gairdner, Numar, RGZLL, SYR01, TAM407227, TF026, Unicorn, Yan89110, Yerong, YF374, YSM1, YYXT, ZUG293 and ZUG403) were selected to validate the newly developed screening method. Among these genotypes, CPI-71284-48, SYR01 and TAM407227 were wild barleys (provided by Adelaide Barley Breeding Program and Australian Barley Collection Centre), and CXHKSL, RGZLL, TX9425, YSM1, YYXT, ZUG293 and ZUG403 were introduced from China through 'Australia and China collaboration on barley germplasm research' and Australia-China Joint Research Centre for Plant Stress Biology.

Barley seeds were surface-sterilised using 0.5% v/v sodium hypochlorite for 15 min, and then thoroughly washed in running water for 30 min. Seeds were germinated on a floating mesh in 1-L plastic containers with aerated distilled water in dark for 3 days. The seedlings were then grown in nutrient solution in the same containers under natural light (day/night 16/8h), at 24 ± 1˚C. The nutrient solution contained 1.5 mM KNO_3, 1.0 mM $Ca(NO_3)_2$, 0.25 mM $MgSO_4$, 0.25 mM $(NH_4)_2HPO_4$, 0.25 mM $NH_4H_2PO_4$, and 4 mM MES hydrate buffer (modified Rygol-Johnson solution; Shabala *et al.* 2010). Freshly prepared 1 M manganese ($MnSO_4$) stock solution was added to nutrient solution to achieve five concentrations (0, 1.0, 5.0, 10.0 and 20.0 mM). The nutrient solutions were renewed every 2 days. The pH of nutrient solutions was checked and adjusted to 4.8 daily using 1 M HCl or 1 M NaOH. To complement water loss from evaporation and transpiration, distilled water was added to maintain the volume of the solution. The protocol of 1 mM Mn treatment in hydroponics for 10 days was selected to screen 20 barley genotypes for Mn^{2+} tolerance.

3.2.2 Measurement of different traits

The seedlings were harvested and analysed after 10 days of Mn treatments. Chlorophyll content of the oldest fully expanded leaves was determined before harvest, using a SPAD-520 chlorophyll meter (Konica Minolta Sensing Inc., Sakai, Japan). Fresh weight of shoots and roots was recorded after harvest. After drying the samples at 70˚C for 3 days, dry weight of shoots and roots was recorded.

Samples of dry materials were ashed for at 500˚C for 8 h. Ashed samples were dissolved in 8.0 mL 1.0 M HCl. A 0.8-mL aliquot was diluted to 8.0 mL with deionised water (Kalra 2010). The Mn content in the tissues was measured using an atomic absorption spectrophotometer (AAS, Thermo Element MKII-M6; Thermo Electron, Waltham, MA, USA).

3.2.3 Evaluation of waterlogging tolerance

Seeds were sown in stainless steel tanks (200 cm x 100 cm x 85 cm) filled with soil in the 2011 – 2012 growing season at Mt Pleasant Laboratories in Launceston, Tasmania. Three replicates were used, with each replicate having 15–20 plants. Starting from the three-leaf stage (Zadoks score of 13), all the genotypes were subjected to waterlogging (keeping the water level just above the soil surface) for 9 weeks until susceptible genotypes died. A combined score system (leaf chlorosis and plant survival after waterlogging, 0 = all dead and 10 = not affected) was used (Zhou 2011).

3.2.4 Statistical analysis

A completely randomised block design with three replicates was employed throughout the assay. Data were subjected to analysis of correlation and variance (with Fisher protected LSD at 5% or Turkey’s test for comparison among means) using IBM SPSS Statistics 20 (Chicago, II, USA).

3.3 Results

3.3.1 Waterlogging tolerance of selected genotypes

The 20 selected barley genotypes showed significant differences in waterlogging stress tolerance (Fig. 3.1). Among these genotypes, Naso Nijo, Franklin and Unicorn were the most sensitive. At the end of waterlogging treatment, most plants of these three genotypes were dead. In contrast, the most tolerant genotype, TAM407227, survived and even continued to grow well under waterlogging conditions. Some other genotypes, such as Yerong, CM72, Yan89110 and RGZLL, also showed good tolerance to waterlogging.

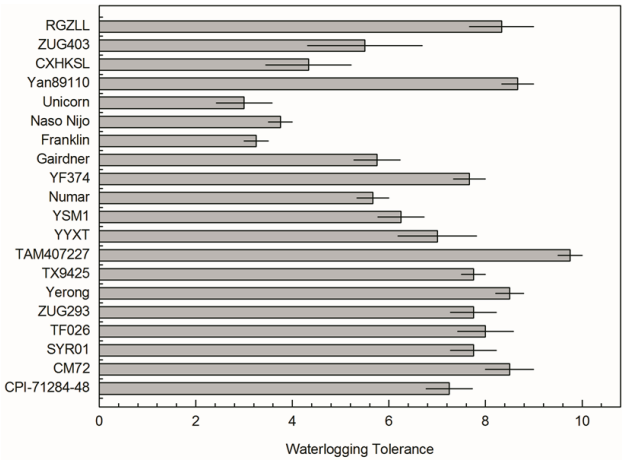

Fig. 3.1 Waterlogging tolerance of different genotypes. 0 = all dead, 10 = not affected, means ± se (n = 15–20 plants of each genotype).

3.3.2 Development of the screening method

Effect of Mn^{2+} on plant growth

The presence of Mn^{2+} in the growth solution significantly influenced seedling growth of Naso Nijo and TX9425, in a dose-dependent manner (Fig. 3.2A and B). Shoot growth was reduced at high Mn^{2+} concentration (leaves were small and curled). At 1 mM Mn^{2+}, root length of Naso Nijo and TX9425 decreased by 28 and 38%, respectively (Fig. 3.2C). The root growth of both genotypes was almost completely inhibited at ≥10 mM Mn^{2+} (Fig. 3.2E). There was no significant difference between these two genotypes in root nor shoot growth inhibition caused by Mn^{2+} (Fig. 3.2D and E; $P > 0.9$).

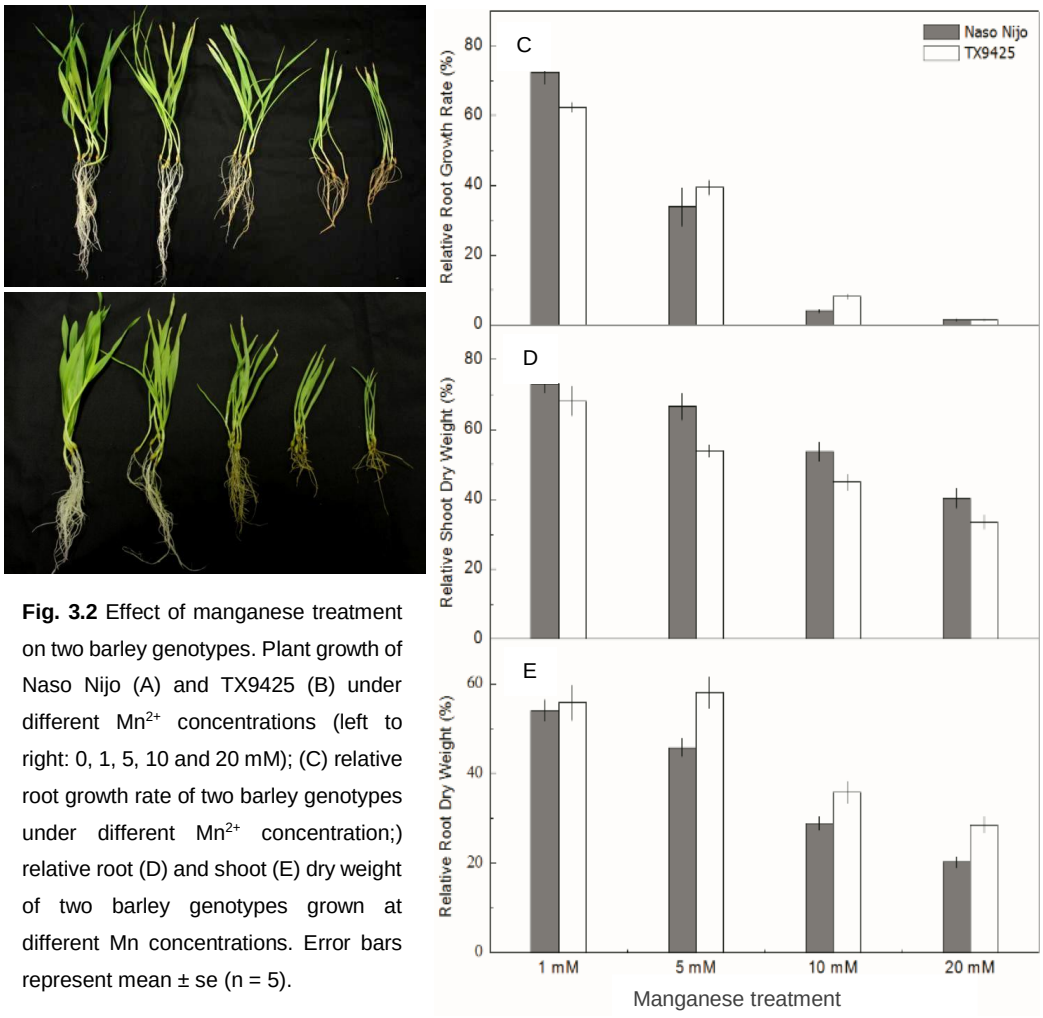

Fig. 3.2 Effect of manganese treatment on two barley genotypes. Plant growth of Naso Nijo (A) and TX9425 (B) under different Mn^{2+} concentrations (left to right: 0, 1, 5, 10 and 20 mM); (C) relative root growth rate of two barley genotypes under different Mn^{2+} concentration;) relative root (D) and shoot (E) dry weight of two barley genotypes grown at different Mn concentrations. Error bars represent mean ± se (n = 5).

Toxicity symptoms

Visual scores: Marginal chlorosis and necrosis of leaves resulted from Mn^{2+} treatment in Naso Nijo and TX9425, increasing Mn^{2+} concentration in the nutrient solution intensified symptoms of Mn^{2+} toxicity on leaves in both genotypes. Toxicity symptoms were more pronounced in Naso Nijo than TX9425. Two days after plants were exposed to 1 mM or 5 mM Mn^{2+} treatment, brown spots started to appear on old Naso Nijo leaves, spreading from the leaf tip to the base, with chlorosis at the leaf tip. In contrast, only tiny brown spots appeared on the TX9425 leaves at 1 mM Mn^{2+} and slight chlorosis appeared at 5 mM Mn^{2+} 6 days after the treatment.

Chlorophyll content

The first fully expended leaf was used to determine chlorophyll content with a SPAD chlorophyll meter. Given that leaves of both Naso Nijo and TX9425 were small and curled under high Mn stress (10 and 20 mM), only leaves from 0, 1, and 5 mM Mn^{2+} concentrations were used for measuring chlorophyll content. The addition of Mn^{2+} to nutrient solution reduced chlorophyll content of both genotypes, with Naso Nijo showing larger reductions (more than 50%) in chlorophyll content than TX9425 (less than 20%) at 1 and 5 mM Mn^{2+} (Fig. 3.3).

The above experiments showed that 1 mM Mn^{2+} treatment best separated the tolerant genotype (TX9425) from the sensitive one (Naso Nijo). Hence, this concentration was utilised to screen a large number of selected genotypes varying in waterlogging stress tolerance.

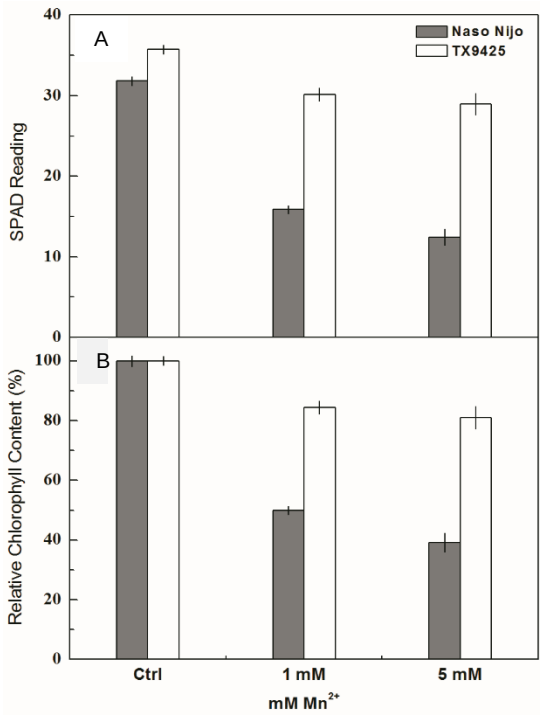

Fig. 3.3 Chlorophyll content of two barley genotypes (Naso Nijo and TX9425) grown with different Mn concentration. (A) chlorophyll content as SPAD reading; (B) relative chlorophyll content (control = 100%). Values are means ± se (n = 5).

3.3.3 Manganese tolerance of barley genotypes and its relationship to waterlogging tolerance

Chlorophyll content and visual symptoms

Chlorophyll content showed significant ($P \leq 0.05$) differences between tolerant and sensitive genotypes at 1 mM Mn^{2+}. Most of the waterlogging-tolerant genotypes (TX9425, TAM407227, TF026, Yerong, CPI-71284-48 and CM72) retained high chlorophyll content at >60% of control values (Fig. 3.4). In contrast, waterlogging-sensitive genotypes (Franklin, Gairdner and Naso Nijo) showed larger reduction in chlorophyll content with relative SPAD values <35% of control. The relative chlorophyll content in TAM407227, the most waterlogging-tolerant genotype, was up to twice as high as that of Franklin.

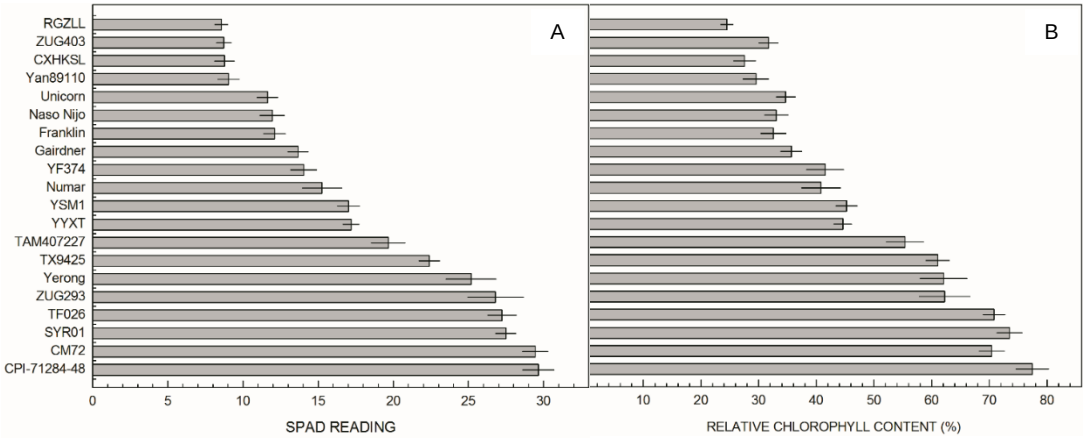

Fig. 3.4 Chlorophyll content of 20 barley genotypes treated with 1 mM Mn in nutrient solution for 10 days. (A) chlorophyll content as the SPAD reading; (B) relative chlorophyll content (control = 100%). Data are means ± se (n = 5).

Fig. 3.5 Toxicity symptoms on the first fully expanded leaf of different genotypes, 6 days after addition of 1 mM Mn. Photos A, B, C and D are tolerant genotypes: CPI-71284-48 (A), SYR01 (B), CM72 (C), Yerong (D). Photos E, F, G and H are sensitive genotypes: Naso Nijo (E), Gairdner (F), Franklin (G), ZUG403 (H).

Significant differences in visual symptoms were also found at 1 mM Mn^{2+} (Fig. 3.5). Only tiny brown spots were observed on the old leaves of CPI-71284-48, SYR01, CM72 and Yerong, whereas toxicity symptoms were more pronounced in sensitive genotypes Naso Nijo, Gairdner and Franklin. However, some waterlogging-tolerant genotypes, such as RGZLL and CXHKSL, also had severe Mn^{2+} toxicity symptoms.

Biomass

The addition of 1 mM Mn^{2+} caused a significant reduction in both shoot and root growth of all the genotypes (Fig. 3.6). Two waterlogging-tolerant wild barleys, CPI-71284-48 and SYR01, had higher relative shoot biomass (85%) than waterlogging-sensitive genotypes Franklin and Gairdner (around 70%). However, there was no obvious correlation between shoot growth and waterlogging tolerance (Table 3.1), with several waterlogging-tolerant genotypes (*e.g.* YF374) showing a decrease of almost 30% in shoot growth. There was no significant difference in relative root dry weight among waterlogging-tolerant and waterlogging-sensitive genotypes.

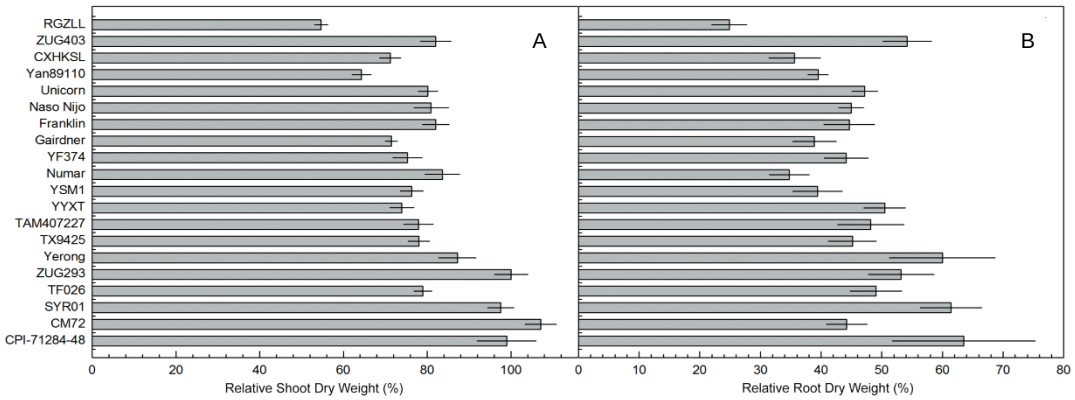

Fig. 3.6 Relative shoot (A) and root (B) dry weight of 20 barley genotypes grown with 1 mM Mn concentration (control = 100%). Each data bar represents a mean ± se (n = 5).

Table 3.1 Correlation coefficients between various growth and physiological parameters of 20 barley genotypes grown at 1 mM Mn.

	Mn content	relative shoot DW	relative root DW	chlorophyll content
relative Shoot DW	-0.82			
relative root DW	-0.68	0.87		
chlorophyll content	-0.68	0.49	0.40	
waterlogging tolerance	-0.18	-0.13	-0.01	0.60

Manganese accumulation in plant tissues

Genotypes showed a significant difference in leaf Mn concentration after the Mn treatment (Fig. 3.7). Three wild genotypes CPI-71284-48, SYR01 and TAM407227 had the lowest Mn concentration (<1,300 $\mu g \cdot g^{-1}$) among the 20 genotypes. Franklin had the highest Mn concentration (>2,700 $\mu g \cdot g^{-1}$) in leaves. The Mn concentration in leaves did not show a relationship with the symptom scores or chlorophyll content in leaves. The Mn concentration in leaves also showed no relationship with waterlogging tolerance, with several waterlogging-tolerant genotypes having relatively high Mn concentration in shoots. For example, Mn concentration in TX9425 (waterlogging-tolerant) reached 2,000 $\mu g \cdot g^{-1}$, which was 500 $\mu g \cdot g^{-1}$ higher than in the waterlogging-sensitive genotype Naso Nijo.

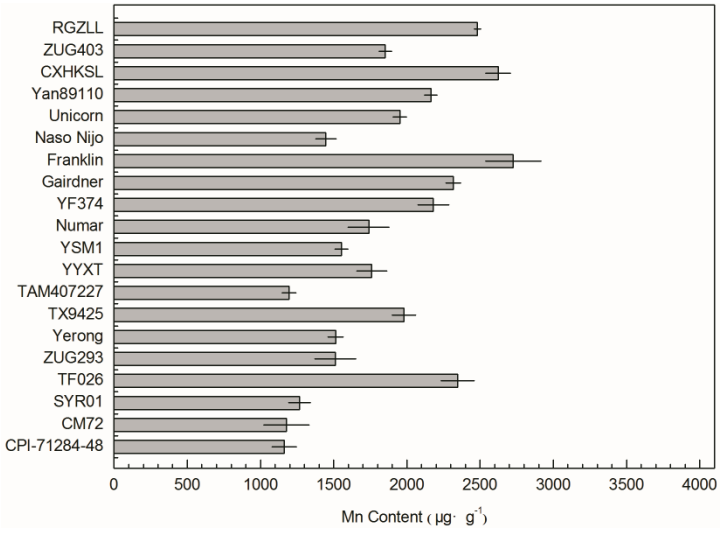

Fig. 3.7 Mn concentration in shoots of 20 barley genotypes grown with 1 mM Mn concentration. Each data bar represents mean ± se (n = 5).

Waterlogging tolerance and manganese^{+} tolerance

Tolerance to Mn^{2+} showed a significant correlation with waterlogging tolerance (Fig. 3.8). All genotypes could be classified into three groups: tolerant to both waterlogging and Mn^{2+} toxicity; tolerant to waterlogging but sensitive to Mn^{2+} toxicity, and sensitive to both waterlogging and Mn^{2+} toxicity. However, some waterlogging-tolerant genotypes showed sensitivity to Mn^{2+} toxicity, indicating the present of other mechanisms of waterlogging tolerance.

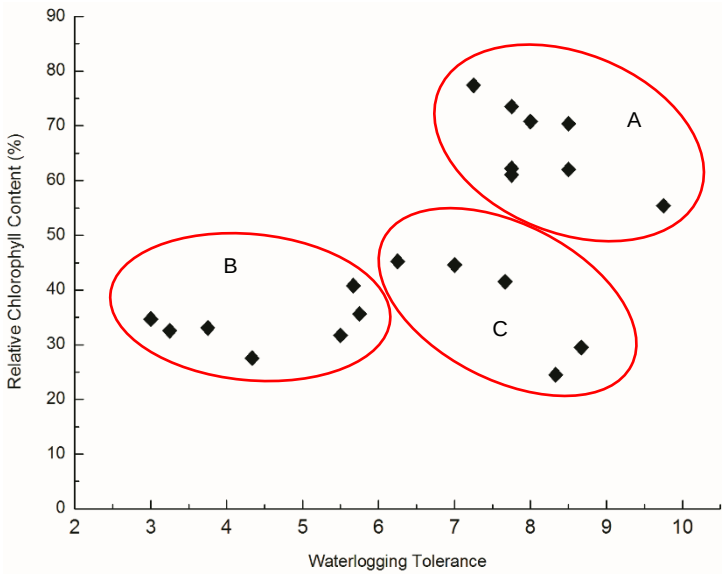

Fig. 3.8 Correlation between waterlogging tolerance and Mn^{2+} tolerance (expressed as relative chlorophyll content) of 20 barley genotypes. (A) waterlogging-tolerant genotypes can maintain high chlorophyll concentration. (B) waterlogging-sensitive genotypes showed a reduction in chlorophyll content. (C) some waterlogging-tolerant genotypes were susceptible to Mn^{2+} toxicity, with relative chlorophyll content lower than 50%.

3.4 Discussion

3.4.1 Manganese toxicity in plants and tolerance mechanisms

Tolerance to elemental ions is one of adaptations by plants to survive in hostile soils, especially acidic ones (McNair *et al.* 2000). Toxicity of Mn^{2+} is considered the second most important factor (after Al^{3+} toxicity) affecting growth in acid soils (Moroni *et al.* 1991a; Khabaz-Saberi *et al.* 2012). Although the expression of Mn^{2+} toxicity varies considerably among plant species, typical symptoms of Mn^{2+}

toxicity are chlorosis and necrosis of leaves (Foy *et al.* 1978; Horiguchi 1987; El-Jaoual and Cox 1998). In our study on barley, the Mn^{2+} toxicity symptoms (similar to those reported in wheat, Macfie *et al.* 1989; Moroni *et al.* 1991a; Khabaz-Saberi *et al.* 2010) developed on most genotypes and became more pronounced with increasing Mn^{2+} concentration in the nutrient solution. Similar to wheat (Scott *et al.* 1998) and rice (Wang *et al.* 2002), toxicity symptoms started on the older leaves and extended to the younger ones.

In our experiment, Mn-sensitive genotypes (e.g. Franklin and CXHKSL) had high, while tolerant genotypes had low, Mn concentrations in shoots. However, some exceptions were found. The tolerant genotypes TX9425 and TF026 had a higher Mn concentration in leaves than the sensitive genotypes Naso Nijo and Numar, suggesting that the former varieties were efficient in either detoxifying or sequestering relatively large amounts of Mn^{2+} in leaves without a significant impact on metabolism. Thus, it is likely that different Mn^{2+} tolerance mechanisms operate in different tolerant genotypes, i.e. exclusion and internal tissue tolerance mechanisms (Hall 2002; Yang and Chu 2011; Hossain *et al.* 2012).

Exclusion mechanisms prevent Mn^{2+} from entering the cytosol and minimize harmful effects in the apoplast. Tissue tolerance mechanisms allow plants to take up and accumulate Mn^{2+} due to complexation, detoxification and compartmentalisation of Mn^{2+} within the plant. In barley, Mn^{2+} exclusion can be the main tolerance mechanism, with most of the tolerant genotypes (eg. CPI-71284-48, CM72 and TAM407227) having low Mn concentration in leaves. Internal tolerance mechanisms may exist in several other Mn^{2+}-tolerant genotypes (e.g. TX9425 and TF026).

A high-affinity Mn^{2+} transporter has been reported in barley (Pai *et al.* 2005), rice (Sasaki *et al.* 2012) and *Arabidopsis* (Cailliatte *et al.* 2010), and knockout or knockdown of the gene associated with this transporter resulted in decreased Mn^{2+} uptake (Sasaki *et al.* 2012). This high-affinity transporter was specific for Mn^{2+} acquisition and controlled differential Mn efficiency among barley genotypes (Pedas *et al.* 2008). Therefore, understanding the factors modulating expression levels and/or activity of Mn^{2+} transporters may be essential for plant breeding for Mn^{2+} stress tolerance.

3.4.2 Mn^{2+} tolerance and waterlogging tolerance

Waterlogging causes reduction in Mn(IV) oxide and increases the concentration of Mn^{2+} above the plant nutritional requirements (Bailey-Serres and Voesenek 2008; Khabaz-Saberi and Rengel 2010). Tolerance to Mn^{2+} is reported to be one of the mechanisms of waterlogging tolerance in wheat (Khabaz-Saberi and Rengel 2010). In our study on barley, a positive relationship between Mn^{2+} tolerance and waterlogging tolerance was found ($r = 0.60$), with Mn^{2+}-tolerant varieties being tolerant to waterlogging and all waterlogging-sensitive varieties being sensitive to Mn^{2+} toxicity. However, several waterlogging-tolerant varieties, such as CXHKSL, showed sensitivity to Mn^{2+} toxicity, confirming that other (more "traditional") mechanisms such as aerenchyma formation or development of impermeable barriers to radial oxygen loss (Jackson and Armstrong 1999; Pang *et al.* 2004; Colmer and Voesenek 2009) also contribute to the tolerance to waterlogging.

3.4.3 Screening methodology

Accurate phenotyping is crucial in breeding barley varieties for Mn^{2+} tolerance. Hydroponic-based methods have several advantages over soil-based methods (Hoagland and Arnon 1950; Watson *et al.* 2003; Tavakkoli *et al.* 2012), including a better control of background nutrients, pH and the stress level. Different Mn^{2+} concentrations have been used in various studies: in wheat, 0.5 mM (Moroni *et al.* 1991b) and 0.75 mM Mn^{2+} (Khabaz-Saberi *et al.* 2010) were used. In our study on barley, 1 mM Mn^{2+} was found to best separate sensitive from tolerant genotypes.

Variation in plant biomass is usually used as one of the major criteria in screening for stress tolerance (Moroni *et al.* 1991b; Scott *et al.* 1998; Khabaz-Saberi *et al.* 2010). However, in the present study relative root and shoot dry weight did not show significant differences among 20 barley genotypes, probably because only a short-term Mn^{2+} treatment was used in the assay. In contrast, toxicity symptoms and chlorophyll content were found to be the best criteria; the relative chlorophyll content of the most sensitive genotypes was less than half that of tolerant genotypes.

In conclusion, a rapid hydroponic method was developed for screening barley for manganese tolerance. The SPAD measurements of chlorophyll content and

visual symptom scores on plants treated with 1 mM Mn^{2+} for 10 days can be used as reliable criteria for tolerance to manganese toxicity. Both exclusion and internal tolerance mechanisms may be involved in Mn^{2+} tolerance. Tolerance to Mn^{2+} showed a significant positive correlation with waterlogging tolerance. Further studies will be conducted in several specific doubled-haploid populations to determine whether the same genes are responsible for Mn^{2+} and waterlogging tolerance.

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Chapter IV: Major QTL control the tolerance to manganese toxicity in barley (Hordeum vulgare L.)[#]

Xin Huang[1], Yun Fan[1], Lana Shabala[1], Zed Rengel[2], Sergey Shabala[1], and Meixue Zhou[1]

[1]School of Land and Food, University of Tasmania, Hobart, TAS 7001, Australia

[2]School of Agriculture and Environment, The University of Western Australia, Perth, WA 6009, Australia

Abstract

Waterlogging stress disturbs plant metabolism through increased ion (Mn and Fe) toxicity resulting from the changes in the soil redox potential under hypoxic conditions. Our previous study found a significant correlation between the tolerance to Mn^{2+} toxicity and waterlogging stress tolerance in barley, suggesting that waterlogging tolerance could be increased by improving tolerance to Mn^{2+} toxicity. In this study, a doubled-haploid (DH) population from the cross between barley varieties Yerong and Franklin (waterlogging-tolerant and sensitive, respectively) was used to identify QTL controlling tolerance to Mn^{2+} toxicity based on chlorophyll content and plant survival as selection criteria. Four significant QTL for plant survival under Mn^{2+} stress (*QSur.yf.1H*, *QSur.yf.3H*, *QSur.yf.4H* and *QSur.yf.6H*) were identified in this population at the seedling growth stage. Two significant QTL controlling leaf chlorosis under Mn^{2+} stress (*QLC.yf.3H* and *QLC.yf.6H*) were identified on chromosomes 3H and 6H close to *QSur.yf.4H* and *QSur.yf.6H*. The major QTL *QSur.yf.3H*, located near the marker Bmag0013, explaining 21% of the phenotypic variation. The major QTL for plant survival on 3H was validated in a different DH population (TX9425/Naso Nijo). This major QTL could potentially be used in breeding programmes to enhance tolerance to both manganese toxicity and waterlogging.

Keywords

Barley (*Hordeum vulgare* L.), Mn^{2+} toxicity, waterlogging tolerance, QTL mapping.

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4.1 Introduction

Waterlogging is one of the most hazardous natural constraints affecting agricultural crop production. Based on soil moisture or water levels, waterlogging may refer to flooding, submergence, soil saturation, anoxia, or hypoxia (Ahmed *et al*. 2012). Soil waterlogging is generally caused by the prolonged rain or flooding in combination with the poor soil drainage. The yield loss can vary with duration of the stress, soil types and the tolerance of different species and genotypes (Bailey-Serres and Voesenek, 2008; Shabala 2011).

Waterlogging stress affects plant metabolism in multiple ways, with plants showing a broad range of morphological and physiological responses to waterlogging. The phenotypic features associated directly with waterlogging tolerance remain unclear and controversial (Pezeshki *et al.* 1999; Smethurst *et al.* 2005; Teakle *et al.* 2010), even though the severity of leaf chlorosis after waterlogging has been proven to be one of the reliable indicators of waterlogging tolerance (Li *et al*. 2008; Zhou 2011). Other symptoms caused by ion toxicity or accumulation of toxic organic metabolites in roots under waterlogging conditions (Pang *et al*. 2007) may also occur (Khabaz-Saberi *et al.* 2006; Bailey-Serres and Voesenek, 2008; Khabaz-Saberi and Rengel 2010; Shabala 2011). The availability of chemical elements in soils is affected by multiple interacting factors, including soil pH, redox potential, cation exchange capacity, and microbial activity. With the redox potential declining after waterlogging onset, metals such as Mn^{4+} and Fe^{3+} are utilised as alternative electron acceptors when oxygen is depleted, resulting in increased concentrations of soluble Mn^{2+} and Fe^{2+} (Kirk *et al.* 2003) that may exceed plant requirements and cause toxicity. Genotypes with tolerance to Mn^{2+} or Fe^{2+} toxicity generally perform better than sensitive ones in waterlogged soils (Pang *et al.* 2007; Khabaz-Saberi and Rengel 2010; Khabaz-Saberi *et al.* 2012). Enhanced tolerance to multiple ion toxicities was reported to improve wheat performance in waterlogging-affected acid soils (Khabaz-Saberi and Rengel 2010). The significant correlation between tolerance to Mn^{2+} toxicity and tolerance to waterlogging in barley (Huang *et al*. 2015) suggests that plant breeding for tolerance to waterlogging traits may be advanced by targeting the tolerance to Mn^{2+} toxicity.

Similar to other waterlogging-related traits, various Mn^{2+} tolerance mechanisms operate in different tolerant genotypes (Huang *et al*. 2015). Direct selection for Mn^{2+} toxicity is affected by environmental factors and is thus largely ineffective. Marker-assisted selection (MAS) is based on distinct genetic differences that can easily be scored and mapped in most segregating populations (Kearsey and Farguhar 1998). During past decades, many QTL have been reported for tolerance to waterlogging or submergence based on various agronomical and morphological characteristics in different doubled-haploid (DH) populations (Qiu *et* al. 2007; Li *et al.* 2008; Zhou 2011; Zhang *et al*. 2013). However, to the best of our knowledge, no QTL for tolerance to Mn^{2+} toxicity has been reported so far.

In the present study, one DH population was used to identify QTL controlling tolerance to Mn^{2+} toxicity, and the QTL were validated in another DH population. Chlorophyll content and plant survival under Mn^{2+} toxicity were used as the indicators of Mn^{2+} tolerance.

4.2 Materials and methods

4.2.1 Plant materials and growth condition

A total of 177 lines from a DH population originated from the cross between Yerong (waterlogging tolerant) and Franklin (waterlogging sensitive) were used to identify QTL conferring Mn^{2+} tolerance. Another 188 DH lines from the cross between TX9425 (waterlogging tolerant) and Naso Nijo (waterlogging sensitive) were used to validate the QTL identified in the Yerong/Franklin population.

The DH lines and parents were grown as reported previously (Huang *et al.* 2015). Seeds were surface-sterilised using 0.5% v/v sodium hypochlorite for 15 min, then thoroughly washed in running water for 30 min. After 1-day germination in a Petri dish, seeds were transferred to the floating mesh in 60-L (60cm x 40cm x 25cm) plastic containers with nutrient solution. The solution was bubbled with air for the duration of the experiment. The nutrient solution contained 1.5 mM KNO_3, 1.0 mM $Ca(NO_3)_2$, 0.25 mM $MgSO_4$, 0.25 mM $(NH_4)_2HPO_4$, 0.25 mM $NH_4H_2PO_4$, and 4 mM MES hydrate buffer (modified Rygol-Johnson solution) (Shabala *et al*. 2010). Aliquots of freshly prepared 1 M manganese ($MnSO_4$) stock solution were

added to the nutrient solution to attain 1 mM final concentration. Nutrient solutions were replaced every 2 days. The pH of nutrient solutions was adjusted to 4.8 daily using 1 M HCl or 1 M NaOH. Distilled water was added to maintain the volume of the solution to compensate for the loss by transpiration.

4.2.2 Evaluating severity of Mn^{2+} toxicity by SPAD chlorophyll measurements

A combined visual scoring system including plant survival (dead leaf percentage) and leaf chlorosis was used in the trial. The scoring (0 = not affected and 10 = all dead) was conducted after 10 days of Mn^{2+} treatment. Chlorophyll content of the oldest fully expanded leaves was determined using a SPAD-520 chlorophyll meter (Konica Minolta Sensing Inc., Sakai, Japan) as a measure of Mn^{2+} tolerance.

Two independent trials were conducted for each of the two DH populations. Both trial had three replications, with each replicate having three plants. Each replication was placed in single plastic containers, and the genotypes were randomly arranged.

4.2.3 Map construction

The genetic linkage map of the Yerong/Franklin population has been published earlier by Li *et al.* (2008), comprising 496 DArT markers and 28 microsatellite (SSR) markers. For the TX9425/Naso Nijo population, the DH lines and the two parental varieties were genotyped with DArTSeq (http://www.diversityarrays.com/dart-application-dartseq). Due to the large number of DNA markers (~30,000 SNP and DArTSeq markers), markers with the same positions or with greater distortion and missing data were removed from the map construction. These markers were combined with previous genotypic data (DArT and SSR markers) (Xu *et al.* 2012; Wang *et al.* 2014). A total of around 2,500 markers were selected to construct the genetic map.

4.2.4 QTL mapping and statistical analysis

The average values from each experiment were used for the identification of QTL associated with Mn^{2+} tolerance. The software package MapQTL6.0 (Van Ooijen 2009) was used to detect QTL that were first analysed by interval mapping (IM). The closest marker at each putative QTL identified using IM was selected as a

cofactor and the selected markers were used as genetic background controls in the approximate multiple QTL model (MQM). The logarithm of the odds (LOD) value thresholds, used to declare the presence of a QTL, were estimated by performing genome-wide permutation tests using at least 1000 permutations of the original data set for each trait, resulting in a 95% significance at LOD threshold 3.0. Two LOD support intervals around each QTL were established by taking the two positions around the peak that had the LOD values two less than the maximum (Van Ooijen 2009) after performing restricted MQM mapping. The percentage of variance explained by each QTL (R^2) was obtained using restricted MQM mapping implemented with MapQTL 6.0. Graphical representation of linkage groups and QTL was generated by using MapChart 2.2 (Voorrips 2002). All other statistical analyses, including calculation of mean values, standard errors, and analysis of variance (ANOVA), were performed using IBM SPSS Statistics 20 (Chicago, IL, USA).

4.3 Results

4.3.1 Reaction of parents and DH lines to Mn^{2+} toxicity

The presence of a high concentration (1 mM) of Mn^{2+} in the growth solution had a significant effect on plant growth. Occurrence of tiny brown spots was the common symptom on older leaf tips at the seedling growth stage (Zadoks score of 11). The symptoms were later spread over the whole leaf and resulted in pronounced leaf chlorosis. The symptom development was faster on susceptible than tolerant genotypes (data no shown). One day after applying excess Mn^{2+}, the susceptible parents Franklin and Naso Nijo already showed numerous tiny brown spots, and leaf chlorosis occurred 2 days later. At the end of the 1 mM Mn^{2+} treatment, both sensitive parents showed severe leaf chlorosis (score of 7). In contrast, both Yerong and TX9425 showed much better tolerance than Franklin and Naso Nijo. At the end of the Mn^{2+} treatment, only brown spots appeared on the leaves of tolerant parents Yerong and TX9425 with no obvious chlorosis. TX9425 was less tolerant than Yerong (Fig. 4.1).

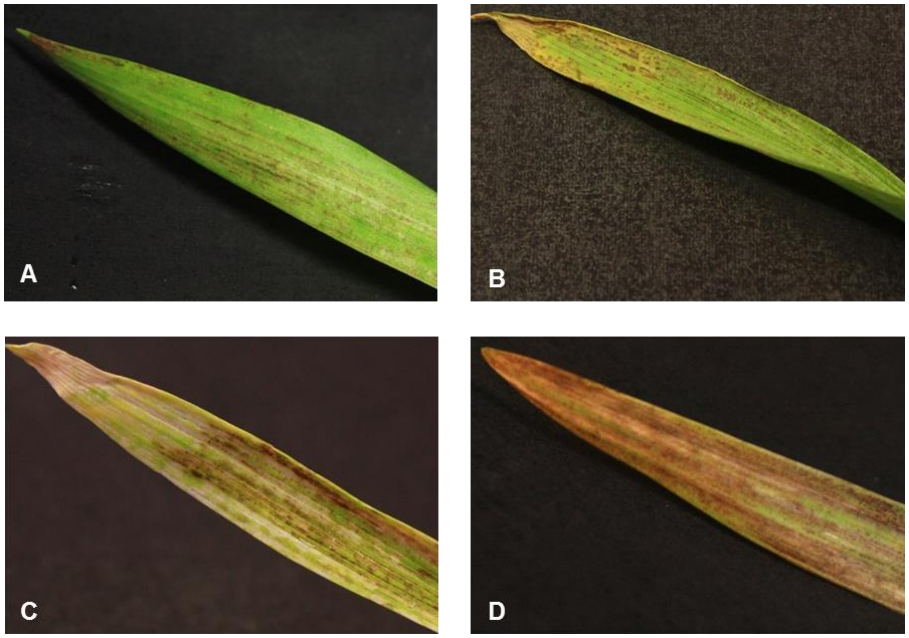

Fig. 4.1 Toxicity symptoms on the oldest fully expanded leaf of the parent genotypes at the end of 1 mM Mn treatment (11-day-old plants). Genotypes tolerant to Mn toxicity: Yerong (A, chlorosis score 2.9), TX9425 (B, chlorosis score 3.2). Genotypes sensitive to Mn toxicity: Franklin (C, chlorosis score 6.6), Naso Nijo (D, chlorosis score 6.7).

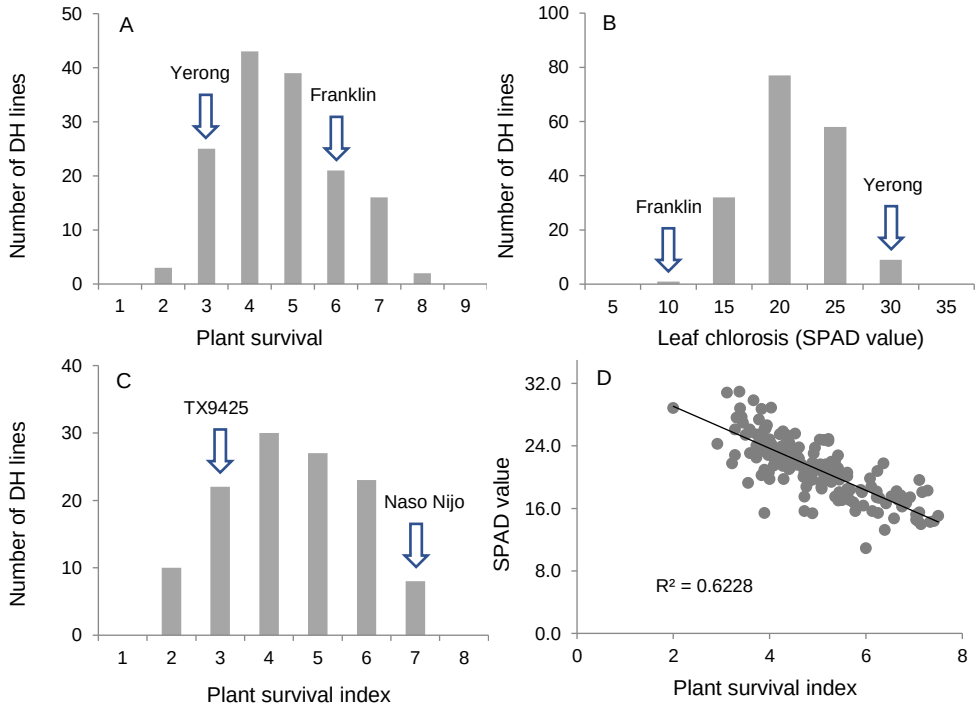

Fig. 4.2 Frequency distribution of Mn^{2+} toxicity tolerance of the Yerong/Franklin DH lines (A, B) and the TX9425/Naso Nijo DH lines (C). (D) Correlation between SPAD values and the plant survival index of both populations. Values are the means of two independent experiments.

A wide variation (2.0-7.5) in plant survival scores index (mainly leaf chlorosis) after the Mn^{2+} treatment was found in the DH lines from both populations (Fig. 4.2A, C). The Yerong x Franklin population also showed a wide variation in chlorophyll content, with the SPAD reading ranging from 14 to 31 (Fig. 4.2B). The plant survival scores in both populations (Fig. 4.2A, C) and the chlorophyll content of the Yerong x Franklin population (Fig. 4.2B) showed normal distribution. Transgressive segregation in the plant survival scores was found in both populations, with some lines surpassing their parents. A significant correlation (R^2 = 0.62) between leaf chlorophyll content and plant survival score was found in the Yerong x Franklin population (Fig. 4.2D).

4.3.2 QTL for chlorophyll content and plant survival under Mn^{2+} stress

Four significant QTL for plant survival under Mn^{2+} stress (*QSur.yf.1H*, *QSur.yf.3H*, *QSur.yf.4H*, and *QSur.yf.6H*) were identified in the Yerong/Franklin DH population at the seedling stage (Table 4.1, Fig. 4.3). The Yerong alleles contributed to the tolerance at all the loci, except for the *QSur.yf.1H* locus where the Franklin alleles improved the plant survival. These four QTL explained up to 40% of the phenotypic variation, with individual QTL explaining between 6.1 and 21% of the phenotypic variation. The major QTL for plant survival (*QSur.yf.3H*) was located on chromosome 3H, with the closest marker being Bmag0013 (Table 4.1).

Two significant QTL (*QLC.yf.3H* and *QLC.yf.6H*) controlling the severity of leaf chlorosis under Mn^{2+} stress were identified on chromosomes 3H and 6H, respectively (Table 4.1, Fig. 4.3). The Yerong alleles were responsible for mild leaf chlorosis (higher chlorophyll content) at both QTL. The major QTL (*QLC.yf.3H*) explained 22% of the phenotypic variation (Table 4.1). This QTL was co-located with *QSur.yf.3H* for plant survival under Mn^{2+} stress (Fig. 4.3). The minor QTL (*QLC.yf.6H*) explained 7.5% of the phenotypic variation. This QTL was located at a similar position to that for plant survival QTL (*QSur.yf.6H*). To test the relationship between chlorophyll content and plant survival scores, QTL analysis was conducted for the chlorophyll content using plant survival scores as covariate, resulting in no significant QTL being identified for the chlorophyll content.

4.3.3 Validation of the major QTL for plant survival in the TX9425/Naso Nijo population

Given that chlorophyll content is closely correlated with plant survival score (Fig. 4.2D), we used plant survival as a suitable proxy to validate the QTL in a different population. In the TX9425/Naso Nijo DH population, only one significant QTL for plant survival under Mn^{2+} stress was identified. The TX9425 alleles increased tolerance to manganese toxicity at *QSur.tx.3H* locus, explaining 15% of the phenotypic variation (Table 4.1). This QTL was colocated with *QSur.yf.3H* (cf. Fig. 4.3).

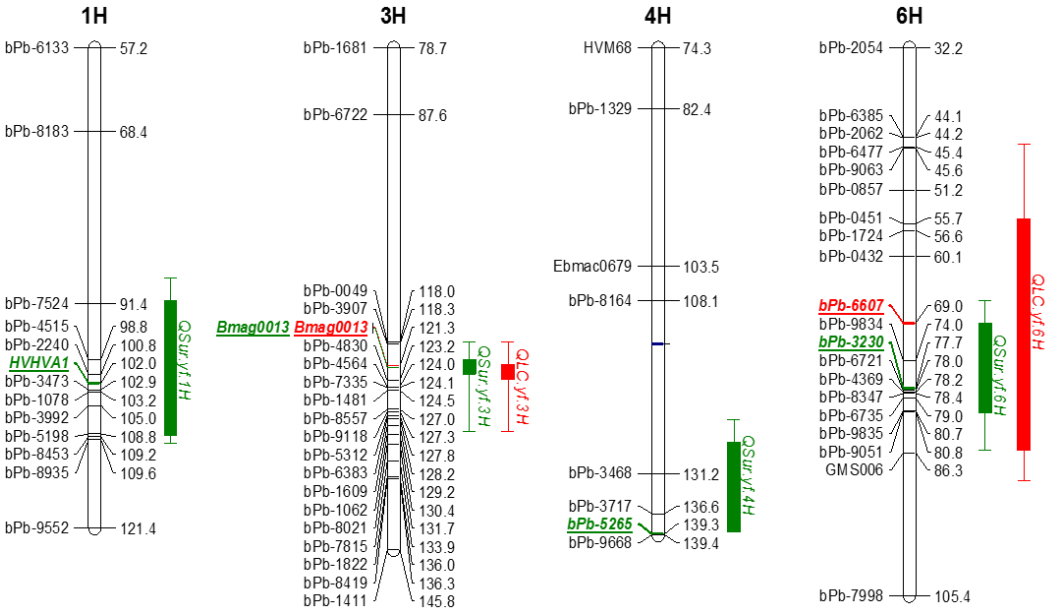

Fig. 4.3 The Yerong/Franklin chromosomes showing the locations of QTL for the traits analysed. Green: QTL for plant survival; red: QTL for leaf chlorophyll content.

Table 4.1 QTL associated with tolerance to Mn identified in the DH populations Yerong x Franklin and TX9425 x Naso Nijo (*yf* and *tn*, respectively, in the QTL name)

Trait	QTL	Chr.	Nearest Marker	One LOD support interval (cM)	LOD	R^2(%)
Leaf Chlorosis (Chlorophyll content)	**QLC.yf.3H**	**3H**	**Bmag0013**	118-122	10.5	22.1
	QLC.yf.6H	6H	bPb-6607	69-80	3.97	7.6
Plant survival	Qsur.yf.1H	1H	HvHVA1	101-102	3.73	6.1
	QSur.yf.3H	**3H**	**Bmag0013**	118-122	9.81	20.9
	QSur.yf.4H	4H	bPb-5265	136-139	3.9	7.7
	QSur.yf.6H	6H	bPb-3230	77-79	3.44	6.8
Plant survival	**Qsur.tn.3H**	**3H**	**3276026**	114-133	3.86	14.6

4.4 Discussion

Waterlogging stress is one of the major abiotic yield-limiting factors in crops, and tolerance to this stress is likely to be a complex trait that is controlled by several mechanisms and complicated by various confounding factors. Because of the low efficiency of direct selection for waterlogging-tolerant plants in the field (Zhou 2010), various indirect criteria have been investigated. Among them, the severity of leaf chlorosis has been used to estimate the capacity for plant survival under waterlogging stress (Li *et al.* 2008; Zhou 2011). In our previous report, the severity of leaf chlorosis showed a significant inverse correlation with Mn^{2+} tolerance, and Mn^{2+} tolerance was significantly correlated with waterlogging tolerance in field conditions (Huang *et al.* 2015). In this experiment, a significant correlation (R^2 = 0.62) between the severity of leaf chlorosis and plant survival under Mn^{2+} stress was also found in the Yerong x Franklin DH population (Fig. 4.2).

An understanding of the genetic background and mechanisms enhancing Mn^{2+} tolerance is crucial in both research and breeding programmes. Several transporters

have been reported to be responsible for Mn accumulation and sequestration (Pittman 2005; Pedas *et al.* 2008; Cailliatte *et al.* 2010; Sasaki *et al.* 2012; Chen *et al.* 2013). However, no QTL has been reported for the tolerance to Mn^{2+} toxicity in barley. Most of the previous studies have targeted Mn^{2+}-use efficiency (Pallotta *et al.* 2000; McDonald *et al.* 2001; Hebbern *et al.* 2005), with a QTL identified on the short arm of chromosome 4H (Pallotta *et al.* 2000). In the study reported here, barley tolerance to Mn^{2+} toxicity was investigated. Four significant QTLs for plant survival (*QSur.yf.1H*, *QSur.yf.3H*, *QSur.yf.4H*, and *QSur.yf.6H*) and two for the severity of leaf chlorosis (*QLC.yf.3H* and *QLC.yf.6H*) under Mn^{2+} stress in the seedling stage were identified in the Yerong/Franklin population. Among them, *QSur.yf.*3H contributed more than 20% of the phenotypic variation for tolerance to Mn toxicity in Yerong/Franklin population, whereas other QTL each explained less than 10%. To our surprise, only one minor QTL controlling plant survival under Mn stress (*QSur.yf.4H*) was located at a similar position as the major QTL for waterlogging tolerance in the Yerong/Franklin population based on the plant survival score after 9 weeks of waterlogging (Zhou 2011). The major QTL on chromosome 3H (*QSur.yf.3H*) and the minor QTL on 6H (*QSur.yf.6H*) are likely to be responsible for waterlogging tolerance at the early treatment stages because QTL for waterlogging tolerance on 3H and 6H were identified after 3 weeks and 2-5 weeks of waterlogging treatment, respectively (Zhou *et al*. 2011).

TX9425 is a landrace from China showing better tolerance to waterlogging stress than Naso Nijo which is a susceptible genotype (Zhou *et al.* 2007, Xu *et al.* 2012). However, the waterlogging tolerance of TX9425 is not as good as that of Yerong (Huang *et al.* 2015). In addition, TX9425 showed lower tolerance to Mn^{2+} toxicity (Fig. 1) and had higher shoot Mn^{2+} concentration under Mn^{2+} stress than Yerong. TX9425 appeared to have a high capacity to sequester Mn^{2+}, maintaining higher Mn content in the shoot than susceptible genotype Naso Nijo (Huang *et al.* 2015). A DH population from the cross of TX9425/Naso Nijo was tested for Mn^{2+} tolerance in the present study, and only one major QTL for plant survival was identified on chromosome 3H. This QTL was at a similar position to the QTL for plant survival and leaf chlorosis (*QSur.yf.3H* and *QLC.yf.3H*) identified in the Yerong/Franklin population. Therefore, it is likely that the better tolerance of

Yerong compared with TX9425 was due to other minor QTL absent in TX9425, i.e. *QSur.yf.1H*, *QSur.yf.3H*, *QSur.yf.4H*, and *QSur.yf.6H*.

Mn^{2+} toxicity can trigger oxidative stress in plant cells (Demirevska-Kepova *et al*. 2004) because high Mn concentration in the cytosol inhibits the non-cyclic photophosphorylation process (Lidon and Teixeira 2000), thus promoting an increase in the production of ROS (specifically, hydroxyl radicals as the most aggressive of all ROS species). This Mn toxicity-induced ROS accumulation may have consequences not only for structural integrity and organisation of the chloroplast lamellae (and, hence, photosynthetic machinery operation), but also for plant ionic relations. Of specific interest is a potential Mn impact on cytosolic K^+ homeostasis. ROS (specifically hydroxyl radicals) are known to be potent activators of various K^+-permeable channels (e.g. Demidchik *et al*. 2003, 2007, 2010; Zepeda-Jazo et al 2001), prompting ROS stress-induced K^+ depletion in the cytosol. Importantly, K^+ is an activator of more than 70 key metabolic enzymes (Dreyer and Uozumi 2011), including those for chlorophyll biosynthesis and photosynthetic CO_2 assimilation. According to the new barley reference genome sequence and annotations (Mascher *et al.* 2017), a candidate gene (*HORVU3Hr1G068040.1*) that codes for *KAT3* was found in the region of the major QTL identified in the present study on chromosome 3H. *KAT3* is as member of the Shaker family of voltage-gated potassium channels (Sharma *et al*. 2013) that are expressed in rice guard cells (Hwang *et al*. 2013).

The other candidate gene *HORVU3Hr1G068200.1* that is also located in this QTL region appears to be more relevant to Mn toxicity tolerance. This gene is identified as *AK369780* in the GenBank database, a member of heavy metal associated domain superfamily (*HMA*). Most of the known HMA transporters were involved in accumulation, transportation, or detoxification of zinc, copper, and cadmium (Takahashi *et al.* 2012; Deng *et al*. 2013; Satoh-Nagasawa *et al*. 2013; Huang *et al.* 2016). However, *HORVU3Hr1G068200.1* has not been assigned any putative function yet. The gene ontology (GO) data suggest that certain portions of the gene are related to heavy metal ion transport (GO:0030001) and metal ion binding (GO:0046870). This gene remains to be investigated further for its possible involvement in the regulation of Mn accumulation and detoxification.

In conclusion, a major QTL for Mn^{2+} toxicity tolerance was identified on chromosome 3H in the Yerong/Franklin DH population and validated in TX9425/Naso Nijo DH population. Three minor QTL were also found in the Yerong/Franklin population. All the QTL likely contribute to waterlogging tolerance in the early growth stage.

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Chapter V: Electrophysiological aspects of barley responses to Mn^{2+} toxicity and hypoxia

Xin Huang[1], Lana Shabala[1], Meixue Zhou[1], Zed Rengel[2], Natsuko I Kobayashi[3] and Sergey Shabala[1]

[1]Tasmanian Institute of Agriculture and School of Agricultural Science, University of Tasmania, P.O. Box 46, Kings Meadows, TAS 7249, Australia.

[2]School of Agriculture and Environment, The University of Western Australia, 35 Stirling Highway, Perth, WA 6009, Australia.

[3]School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, 113-8657, Japan

Abstract

Enhancing Mn^{2+} toxicity tolerance is considered as a novel approach to improve plant's performance in waterlogged soil. The linkage and tolerance-mechanisms are still unclear and controversial. In this work, changes in net K^+, Ca^{2+} and H^+ fluxes, and membrane potential (MP) of root cells were measured from mature zones of barley roots. We showed that Mn^{2+} plays a vital role on maintenance of H^+ pumping activity, with a result in more pronounced membrane hyperpolarisation. The extent of this hyperpolarisation mediated a significant K^+ uptake in roots but not in shoot mesophyll cells, indicating different mechanisms in regulating K^+ homeostasis. Mn-treatment also induced a Ca^{2+} efflux, therein, both steady Ca^{2+} flux and total Ca^{2+} flux showed a significant correlation with Mn toxicity tolerance. Based on the result from isotope tracer measurement which was compared with Mn content in shoot tissues, tolerant genotypes had a same Mn uptake rate as sensitive genotypes but accumulated less Mn in the shoot, suggesting a sequestration strategy in cellular detoxification in roots thus transported less Mn to the shoot. Additionally, a beneficial effect of Mn application was observed under hypoxia conditions by improving roots ability to maintain K^+ uptake that desensitised root to exogenous H_2O_2. Our pharmacological data also showed K^+ selective and non-selective cations channels mediated Mn-induced ion transport across the root membrane, and NADPH oxidase played a substantial role on

regulation of ROS production and K^+ signalling. It is concluded that Mn may be a key factor conferring a better K^+ retention in root under hypoxia condition.

Keywords

Barley (*Hordeum vulgare* L.), Mn^{2+} toxicity, waterlogging stress tolerance, signalling, K^+ retention, ROS

5.1 Introduction

Waterlogging (WL) is a common environmental constraint affecting a large land area and limiting agricultural production worldwide (Setter and Waters 2003) resulting in substantial (up to 80%) (Shaw *et al*. 2013) yield losses to crops. Waterlogging is a complex constraint, altering plant metabolism in multiple ways. The traditionally targeted traits related in plant breeding for waterlogging tolerance include mechanisms dealing with oxygen availability such as those preventing oxygen loss from non-meristematic root tissues or improving oxygen transport to, or storage in, the root (Jackson and Armstrong 1999). At the same time, many metal oxides, including Fe^{3+} and Mn^{4+}, perform as alternative electron acceptors in flooded soils, resulting in an increased concentration of Fe^{2+} and Mn^{2+} under flooding conditions (Pezeshki and DeLaune 1998; Bailey-Serres and Voesenek 2008; Igamberdiev and Hill, 2009; Khabaz-Saberi and Rengel, 2010). However, oxygen availability or ability to retain oxygen in root is given priority in breeding program, while soil elemental toxicity was essentially neglected, even the importance of this toxicity was emphasised on many occasions (Khabaz-Saberi *et al.* 2006; Shabala 2011). Most of the previous studies on barley were targeted at manganese use efficiency (Pallotta *et al.* 2000; McDonald *et al.* 2001; Hebbern *et al.* 2005). Nevertheless, genotypes with ability to cope with Mn^{2+} or Fe^{2+} toxicity showed better performance in waterlogged soil (Pang *et al.* 2007; Khabaz-Saberi and Rengel 2010; Khabaz-Saberi *et al.* 2012). Tolerance to multiple ion toxicity as an innovative approach to cope with waterlogging stress was reported to improve root and shoot growth in waterlogging-affected acid soils in wheat (Khabaz-Saberi and Rengel 2010). Our previous report also indicated that tolerance to Mn^{2+} toxicity was positively correlated with waterlogging stress tolerance in barley, wherein both

cellular detoxification and avoidance from acquisition contributed to this tolerance (Huang *et al*. 2015). However, specific details of their coordination and the relative contribution of these components towards tolerance to manganese toxicity in barley have not been fully revealed (Rengel 2000; Pittman 2005).

Manganese is taken up and transported into the cell only in its reduced form of Mn(II) or divalent cation Mn^{2+}. Numerous divalent cation transporter family such as Fe^{2+} and Ca^{2+} transporters have the ability to transport Mn into the plant cells (Pittman 2005). IRT1 was revealed to have an ability to transport multiple metal ions including Mn^{2+}, and it was suggested as the predominant pathway for Mn transport during Fe deficiency in barley (Pedas *et al*. 2008). Additionally, NRAMP members are involved in numerous functions including uptake, translocation, and intracellular transport of transition metals (Nevo and Nelson 2006). NRAMP1 and NRAMP5 were also reported to contribute to Mn uptake into root cells (Cailliatte *et al*. 2010; Sasaki *et al*. 2012). Interestingly, OsNRAMP1 was not responsible for Mn uptake in rice roots, where NRAMP5 accounted for this role (Ishimaru *et al*. 2012). Under flooding condition, a reduction of Mn^{2+} concentration occurred in the shoot in NRAMP5 knock-out lines (Sasaki *et al.* 2012), suggesting NRAMP5 might play a significant role in Mn uptake in waterlogged soil. The removal of Mn from the cytoplasm constitutes a substantial tolerance mechanism. In *Arabidopsis*, AtCAX2 and AtCAX4 cation/proton antiporters conferred this trait by mediating transport of Mn into the vacuole (Cheng *et al*. 2002; Schaaf *et al*. 2002; Koren'kov *et al*. 2007). MTP8 was reported to have ability to sequestrate excess Mn into vacuole through vacuolar membrane when identified in *Arabidopsis* (Delhaize *et al*. 2003). Another CDF family member MTP11 presenting in pre-vacuolar or Golgi vesicles, was also revealed to be responsible for Mn tolerance (Delhaize *et al*. 2007).

It has been shown that plant tolerance to many environmental stresses such as salinity (Chen *et al*. 2005; Cuin *et al.* 2011), drought (Cakmak and Engels 1999; Hu and Schmidhalter 2005), and oxidative stress (Demidchik 2014) are ultimately related to the plant's ability to retain K^+ in the cell's cytosol. Potassium is the most abundant cationic component of plants, composing up to 10% of total plant dry weight (Wylupek *et al.* 2014). K fulfils important physiological functions and is necessary for enzyme activation, stabilisation of protein synthesis, neutralisation of negative charges on proteins, maintenance of cytoplasmic pH, osmotic regulation

and growth (Shabala 2003; Dreyer and Uozumi 2011; Shabala and Pottosin 2014). The role of cytosolic K^+ retention in plant tolerance to hypoxia and flooding is understood much less than that to salinity (Shabala and Cuin 2008; Shabala and Pottosin 2014), and not much is known about disturbance to K^+ ionic homeostasis caused by Mn stress. However, Mn toxicity can trigger oxidative stress in plant cells, inducing generation of reactive oxygen species (ROS), mainly OH, the most reactive ROS and interact directly with most biomolecules (Lynch and Clair 2004; Halliwell and Gutteridge 2015). At the same time, Mn is known to be an essential activator of some superoxide dismutase (SOD) isoforms that are present in both mitochondria and peroxisomes of the plant cell (Alscher *et al.* 2002) and detoxify ROS species. Thus, the relation between Mn concentration in the cell, ROS levels, and their impact on K^+ homeostasis are rather complicated. Filling this gap in our knowledge was the main aim of this study.

5.2 Materials and methods

5.2.1 Plant materials and growth condition

Nine barley (*Hordeum vulgare* L.) genotypes were selected to conduct non-invasive ion flux measurement. Franklin, Gairdner, Unicorn and Naso Nijo are Mn sensitive genotypes; CM72, TX9425 and Yerong are Mn tolerant genotypes, YSM1 is a moderate tolerant genotype; and CPI-71284-48 (CPI) is a Mn-tolerant wild type (Huang *et al.* 2015). Barley seeds were surface-sterilised using 0.5% v/v sodium hypochlorite for 15 min, and then thoroughly washed in running water for 30 min. Seeds were germinated on a floating mesh in plastic containers with aerated basic-salt medium solution (BSM, containing 0.5 mM KCl, 0.1 mM $CaCl_2$, pH 5.6 unbuffered) in the dark (at 24 ± 1˚C) for 3 days. Root length was 50 to 80 mm at the third day.

For long-term treatment experiments, seeds were germinated in the same conditions as described above. Seedlings were then grown in a nutrient solution containing 1.5 mM KNO_3, 1.0 mM $Ca(NO_3)_2$, 0.25 mM $MgSO_4$, 0.25 mM $(NH_4)_2HPO_4$, 0.25 mM $NH_4H_2PO_4$, and 4 mM MES hydrate buffer (modified Rygol-Johnson solution; Shabala *et al.* 2010; Huang *et al.* 2015). Four different

treatments were applied in the nutrient solution as follows: aerated (control); 1 mM Mn; hypoxia (with 0.2 % agar bubbled with N_2); and hypoxia plus 1 mM Mn.

For isotope tracer experiments, six barley genotypes (Gairdner, Naso Nijo, Franklin, Yerong, CM72 and CPI-71284-48) were selected. Surface-sterilisation step is as same as mentioned above. Experiments were conducted in a plant growth chamber at 24 ± 1˚C, Light/Dark = 12h/12h. Barley seeds were germinated on the wet paper towel for 1 day, then transferred to the plastic net floating on the distilled water for another 2 days. Two different Mn concentration (0.2 µM and 1 mM, respectively) were applied for 1 day. Seedlings grown in 0.2 µM Mn concentration set were used as a control group.

5.2.2 MIFE non-invasive ion flux measurement

Net ion fluxes were measured using non-invasive ion-selective vibrating microelectrodes (the MIFE technique, University of Tasmania, Hobart, Australia). The principles of MIFE ion fluxes measurements are described in full elsewhere (Shabala *et al.* 1997; Shabala 2006), and all the details of microelectrodes fabrication and calibration are available in a previous publication (Pang *et al.* 2007). Liquid ionic exchangers used in this work were commercially available ionophore cocktails (Fluka; catalog no.95297 for H^+; 21048 for Ca^{2+}; 60031 for K^+).

5.2.3 Experimental protocol

The 1 mM Mn^{2+} concentration treatment was chosen for most of experiments. One hour before the measurement, a seedling was placed into a plexiglass measuring chamber as described in Pang *et al.* (2007), and 8 mL BSM solution (0.5 mM KCl, 0.1 mM $CaCl_2$, pH 4.8 with 4 mM MES) were added.

In the transient experiments, steady-state fluxes (detail can be found below) were measured for 5 min, then 1 mL of BSM solution containing an appropriate concentration of Mn^{2+} was added into a chamber with 10-mL volume to achieve final concentration 1 mM Mn^{2+}. The measurement continued for further 30 min. All pH of the solution which contained 4 mM MES buffered was adjusted to 4.8. About 0.5 to 1 min, was required for solution to maintain unstirred condition. This period of time was discarded from analysis and appears as a gap in the figures.

For measuring long-term stress effects on roots, ion fluxes were measured after 5 d of plant exposure to the appropriate stress (Mn, hypoxia and hypoxia plus Mn). Cut roots were placed into the 10-mL chamber as described above, and steady state fluxes were measured for several minutes.

The third type of experiments investigated effects of Mn on ROS sensitivity of root cells. For doing this, roots exposed to appropriate treatment (control, 1 mM Mn, hypoxia and 1 mM Mn plus hypoxia) for 5 days were further treated with 10 mM H_2O_2 followed by measuring transient flux kinetics.

The most of electrophysiological experiments on roots was performed on the Mn-sensitive variety 'Franklin', which was expected to have stronger responses to excess Mn^{2+} based on previous phenotypically assays (Huang *et al*. 2015). Therefore, most of results below regard to 'Franklin' root unless specified otherwise.

For detecting ion flux responses from leaf mesophyll, the second newest leaves were harvested from 4-week-old barley plant (Franklin) and cut into several square pieces (7 mm x 7 mm). Leaf segments were conditioned in BSM solution with pH at 5.6 for 24 hours and then ion fluxes were measured from the leaf mesophyll as described in Wu *et al.* (2015).

5.2.4 Pharmacology

Pretreatment with inhibitors was conducted on the root of the cultivar Franklin. The following chemicals (Table 5.1) were used to modify the activity of selected PM transporters. Diphenyleneiodonium (DPI, an NADPH oxidase inhibitor, Morré 2002), tetraethylammonium chloride (TEA, extracellular K^+ channel blocker, Murphy *et al.* 1999), $GdCl_3$ (NSCC blocker, Demidchik *et al.* 2002), $LaCl_3$ (plasma membrane Ca^{2+} channel blocker, Huang *et al.* 1994), thapsigargin (TG, endomembrane Ca^{2+} ATPase blocker, Ordenes *et al.* 2002), and sodium orthovanadate (vanadate, a blocker of plasma membrane H^+-ATPases). These inhibitors were mixed with BSM (with pH 4.8, 4 mM MES) to achieve their final concentration, which were as follows: DPI, 20 µM, TEA, 5 mM, $GdCl_3$, 100 µM, $LaCl_3$, 500 µM, TG, 5 µM, and Vanadate, 1 mM. After 1-hour pretreatment with the appropriate inhibitors, transient ion flux responses to 1 mM Mn^{2+} were

measured. Transient changes response to 1 mM Mn without pretreatment of inhibitors were set as control group.

Table 5.1 Function and concentration of different inhibitors used in experiment

Inhibitor	Function	Conc.	Ref.
DPI	NADPH oxidase inhibitor	20 µM	Morre´ (2002)
TEA	Extracellular K^+ channel blocker	5 mM	Murphy et al. (1999)
Gd^{3+}	NSCCs blocker	100 µM	Demidchik et al. (2002)
La^{3+}	Plasma membrane Ca^{2+} channel blocker	500 µM	Muir et al. (1997)
Vanadate	Plasma membrane ATPases inhibitor	1 mM	Palmgren (2001)
TG	Endomembrane Ca^{2+} ATPase blocker	5 µM	Ordenes et al. (2002)

5.2.5 Membrane potential measurements

Intact barley roots were immobilised in a measuring chamber. Experimental conditions were the same as those for ion flux measurement. 60 min was required for adaption of root in measuring chamber. Measurements of the electrical potential difference (V_m) across the root-cell membranes were conducted in the root mature zone as described by Cuin and Shabala (2005).

5.2.6 ^{54}Mn tracer measurements

Two different concentration of ^{54}Mn (PerkinElmer) were added, 5.2 kBq/ml to 0.2 µM Mn solution and 13 kBq/ml to 1 mM Mn solution. Roots were bathed in the ^{54}Mn solution for 1 h in the plant growth chamber (same as above), then rinsed in the ice-cooled water containing 0.2 µM and 1 mM Mn for another 10 min. Roots and shoots were harvested and weighted.

Samples were put in a plastic vial. Gamma-radiation was detected using a well-type NaI (Tl) scintillation counter (ARC-300, Aloka, Tokyo, Japan) with the counting window set at 585-1085 keV. Mn uptake rate and shoot/root Mn

concentration ratio were calculated. These measurements were conducted by our collaborators at the University of Tokyo in Japan.

5.2.7 RNA extraction and RT-qPCR

Barley genotype Franklin was grown in BSM for 3 d and then transferred into BSM containing 1 mM $MnSO_4$ for 2 h and 24 h respectively. Total RNA was extracted from ~100 mg of root tissue using MiniBEST Universal RNA Extraction Kit (TaKaRa, cat. #9767). First-strand cDNA was synthesised using PrimeScript™ Synthesis Kit (TaKaRa). Relative transcript levels of two genes (*HvHAK4*, *HvHAK5*) and a reference gene (*HvActin*) were assayed by real-time qPCR analysis using Roche LightCycler 480 II system. RT-qPCR conditions were as follows: pre-incubation, 95 ℃ for 30 s; amplification, 95 ℃ for 5 s, 60 ℃ for 30s (40 cycles); melting curve, 95 ℃ for 5s, 65 ℃ for 1 min. Amplified products were detected using iTaq™ Universal SYBR Green Spermix (Bio-Rad). Each data point was determined in triplicate in each sample (three replicates) and presented as mean mean ± SE (n = 9). For the detailed primer information, please refer to Table 5.2. The double delta Ct (ΔΔCt) method (Livak and Schmittgen 2001) was used for PCR array data analysis. The transcript level of *HvHAK4*, *HvHAK5*, and *HvActin* genes in root tissues in barley variety Franklin applying control and 1 mM Mn condition were quantified using RT-qPCR.

Table 5.2 The primers used in the gene expression study and their respective amplication size.

Gene Name	Forward Primer	Reverse Primer	Amplication Size (bp)
HvHAK4	ACTCCATCCCAG GACCTGTA	CCACCTCGATGT GTGGACAA	161
HvHAK5	GTGACGGTGTGC TAACTCCA	GCCAAAGCGCT GAACAAGAA	143
HvActin	GACTCTGGTGAT GGTGTCAGC	GGCTGGAAGAG GACCTCAGG	Reference Gene

5.3 Results

5.3.1 Mn-induced ion flux kinetics in root and mesophyll barley cells

Addition of 1 mM Mn^{2+} to the bath solution resulted in altered fluxes of K^+, Ca^{2+} and H^+ across the plasma membrane of root epidermal cells (Fig. 5.1). Manganese treatment caused a transient increase in net Ca^{2+} and H^+ fluxes (Fig. 5.1b and c, respectively) that gradually recovered in about 20-25 min. The effect of Mn^{2+} on net K^+ flux was more complex, causing a brief shift towards more negative values that lasted for only a few minutes and was then flowed by Mn-induced increase in K^+ uptake. As such, several quantitative characteristics were introduced (Fig. 5.1a). This included: (i) initial flux (IF/I-Flux, mean magnitude of first 5 min); (ii) peak K^+ flux (PF/P-Flux, the highest or lowest magnitude which was relative to the initial flux after applying treatment); (iii) steady K^+ flux (SF/S-Flux, the mean magnitude of last 3 – 5 min which was relative to initial flux); (iv) total K^+ flux (TF/T-Flux, total magnitude during 25 min after applying treatment which was relative to total magnitude of first 5 min).

Net Mn-induced ion flux responses from roots cells were strikingly different from those measured from leaf mesophyll (Fig. 5.2). Here, application of 1 mM Mn^{2+} caused a significant K^+ efflux that cells recovered gradually to the initial state level in 15 – 20 mins (Fig. 5.2a). A similar trend was also detected for net Ca^{2+} fluxes (Fig. 5.2b). Contrary to roots, Mn-induced stimulation of H^+ pumping (net efflux) was prolonged (Fig. 5.2c). There was no significant difference among 100 µM, 250 µM and 500 µM Mn concentration. 1000 µM Mn concentration caused almost 500 nmol m^{-2} s^{-1} reduction of K^+ uptake (Fig. 5.2d). The repress of leaf mesophyll cells influenced by Mn treatment showed a clear dose-dependency (Fig. 5.2d-f).

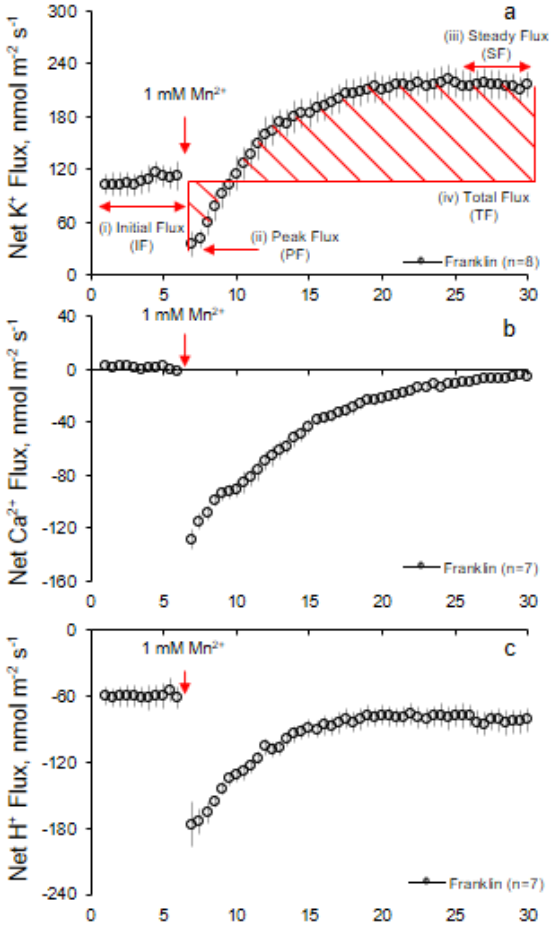

Fig. 5.1 A typical example of transient change of (a) K^+, (b) Ca^{2+} and (c) H^+ fluxes upon the addition of 1 mM Mn to mature zone of barley root of variety Franklin. The arrow indicates commencing of the treatment. Indicators in (a) are explained as follows: (i) Initial flux; (ii) Peak flux; (iii) Steady flux; (iv) Total flux. Data present as mean ± se (n = 7 - 8).

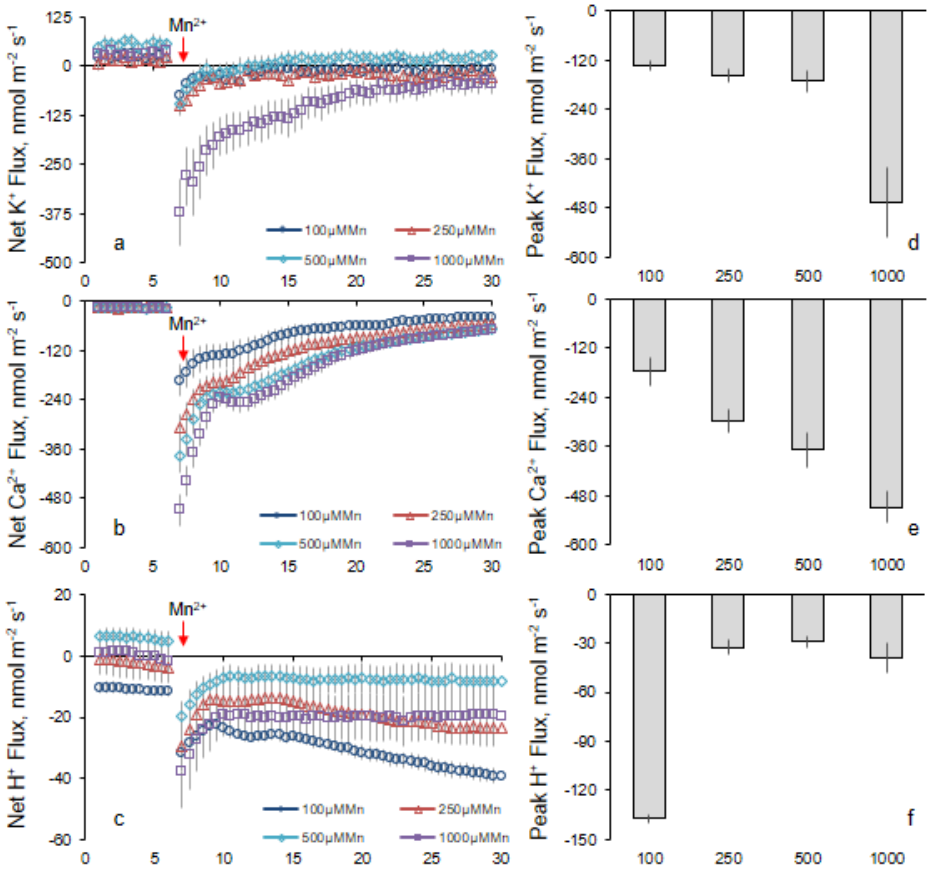

Fig. 5.2 Transient changes of (a) K^+, (c) Ca^{2+} and (e) H^+ fluxe in leaf tissue of Franklin applied four different Mn concentration (100 µM, 250 µM, 500 µM and 1000 µM) in leaf tissue. Data present as mean ± se (n = 6 - 8).

5.3.2 Genotypical difference in transient fluxes

We next compared Mn-induced ion flux responses from a range of barley accessions (9 additional genotypes) that differed in their Mn tolerance. Net K^+ influx was measured under control (initial-state, initial 5 min) condition, ranged from 105 to 265 nmol m^{-2} s^{-1} (Fig. 5.3a and b). Addition of 1 mM Mn^{2+} triggered an instantaneous reduction of K^+ uptake in all genotypes. However, it recovered quickly within 5 to 8 min and followed by a prolonged significant increase in net K^+ uptake, then reached steady-state in about 20 min (Fig. 5.3a). Among these ten genotypes, most of the Mn-sensitive genotypes (Gairdner, Naso Nijo and Unicorn) had a higher initial flux (at around 200 nmol m^{-2} s^{-1}), while tolerant genotypes

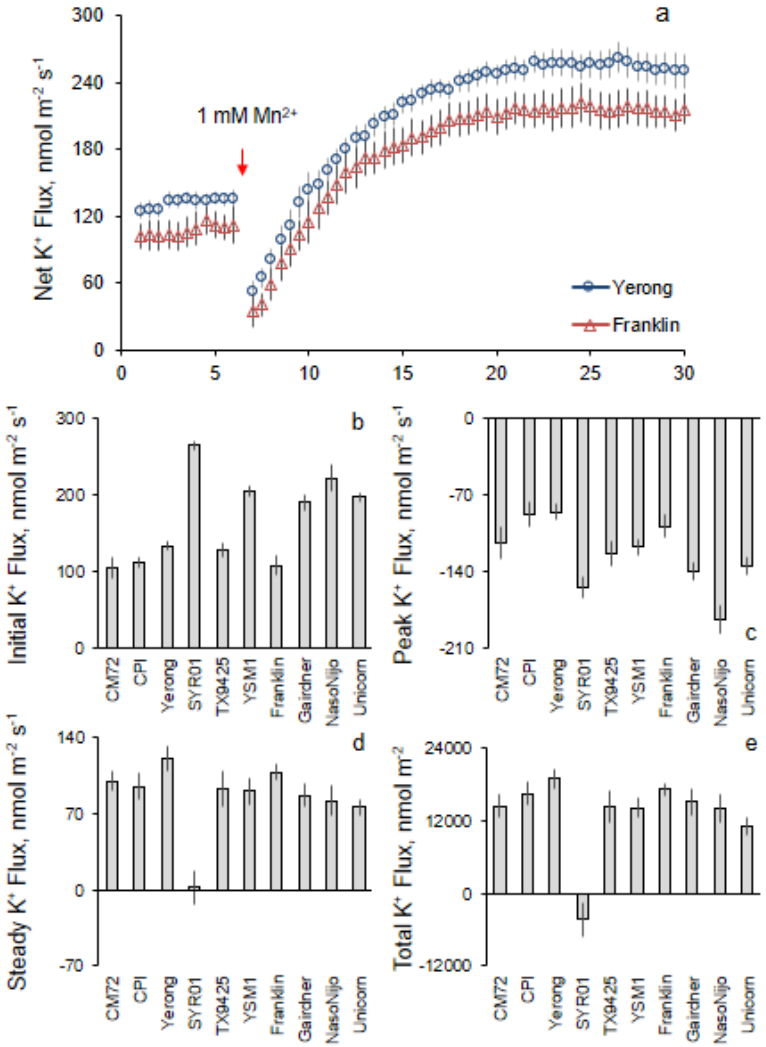

Fig. 5.3 (a) A typical example of transient changes of K^+ fluxes upon the addition of 1 mM Mn to barley root mature zone of Yerong (tolerant) and Franklin (sensitive). The arrow indicates commencing of the treatment. The magnitude of K^+ fluxes was determined as follows: (b) Initial K^+ flux; (c) Peak K^+ flux; (d) Steady K^+ flux; (e) Total K^+ flux. Data present as mean ± se (n = 6 – 10).

Table 5.3 Correlation between K^+ fluxes and several parameters

	Mn Content	Mn-tolerance	SPAD	Relative.SPAD	Fresh Weight (Shoot)	Fresh Weight (Root)	Dry Weight (Shoot)	Dry Weight (Root)
P-Flux	-0.2542	-0.0450	-0.1608	0.1164	0.2143	-0.1225	0.1660	-0.0059
S-Flux	-0.0035	-0.3801	-0.5592	-0.3444	0.1025	-0.1643	-0.1382	-0.0411
Total K+	0.3503	-0.1094	-0.1032	-0.3633	-0.3518	-0.1443	-0.4758	-0.2243

* If r≥0.632, P<0.05

CM72, Yerong and wildtype CPI remained around 100 nmol m^{-2} s^{-1} K^+ influx (Fig. 5.3b). Similarly, Gairdner, Naso Nijo and Unicorn had more than 140 nmol m^{-2} s^{-1} K^+ efflux after applying 1 mM Mn treatment while tolerant genotypes could maintain a low K^+ leakage at around 80 nmol m^{-2} s^{-1} (Fig. 5.3c). Most of the

genotypes were able to achieve a higher K^+ influx at steady state, resulting in better K^+ uptake (Fig. 5.3d, e). However, no significant trends in Mn-induced net K^+ fluxes were measured (Fig. 5.3b-e) in any characteristics; this is further supported by the correlation analysis (Table 5.3).

Likewise, Mn also caused an immediate and substantial Ca^{2+} efflux from barley roots, which returned to its initial state during last 5 to 10 min, varying in different genotypes (Fig. 5.4a). Net zero Ca^{2+} flux was measured under control (initial-state) conditions from barley roots and was not significantly different amongst all genotypes (Fig. 5.4b). Tolerant genotypes Yerong and CM72 showed stronger response when treated with 1 mM Mn^{2+}, resulting in a significant Ca^{2+} efflux compared with sensitive genotypes Naso Nijio and Unicron. However, wildtype CPI maintained a low Ca^{2+} efflux which was responding to Mn treatment, at around 90 nmol m^{-2} s^{-1} (Fig. 5.4c). The genotypic difference between sensitive and tolerant varieties was also obvious for steady Ca^{2+} flux (Fig. 5.4c) and total Ca^{2+} leakage (Fig. 5.4e). Most of the tolerant genotypes maintained higher Ca^{2+} efflux than sensitive genotypes and consequently led to substantial total Ca^{2+} leakage, almost 2-fold higher than sensitive genotypes (Fig. 5.4d and e). Overall, both steady Ca^{2+} flux and total Ca^{2+} flux showed a significant correlation with plant manganese toxicity tolerance (Table 5.4).

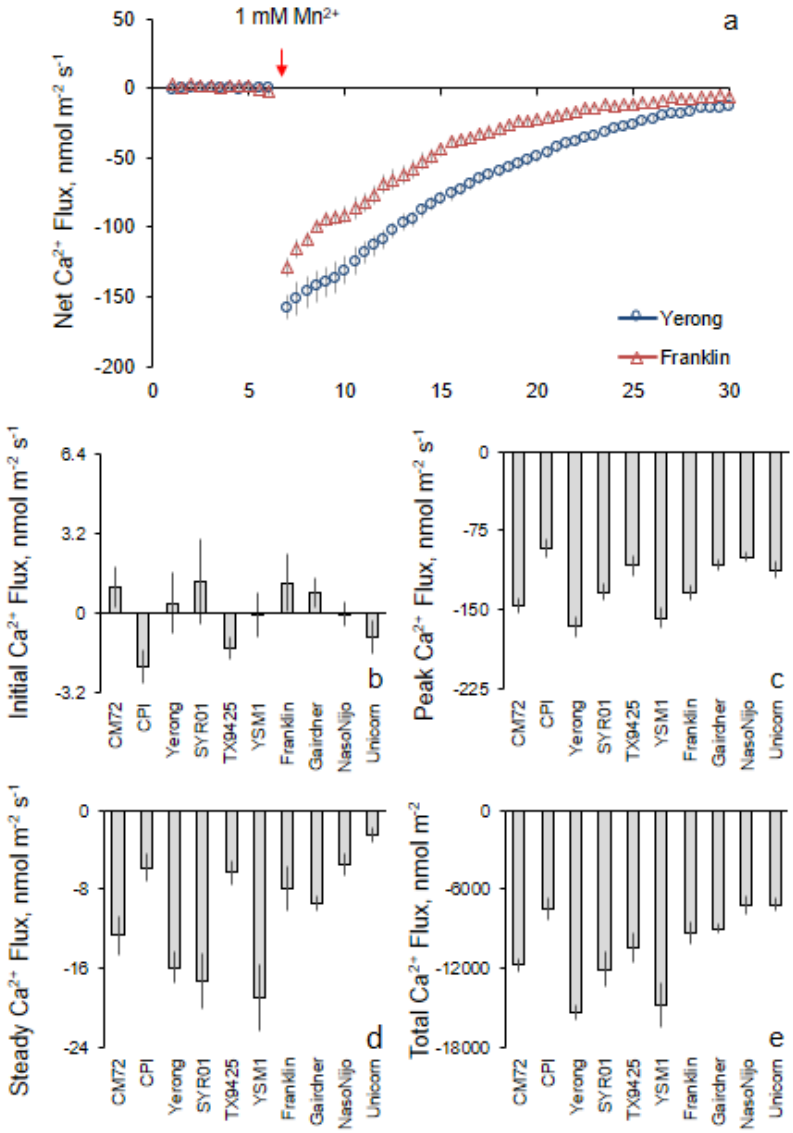

Fig. 5.4 (a) A typical example of transient change of Ca^{2+} flux upon the addition of 1 mM Mn
to barley root mature of Yerong (tolerant) and Franklin (sensitive). The arrow indicates commencing of the treatment. The magnitude of Ca^{2+} flux was determined as follows: (b) Initial Ca^{2+} flux; (c) Peak Ca^{2+} flux; (d) Steady Ca^{2+} Flux; (e) Total Ca^{2+} flux. Data present as mean ± se (n = 6 – 10).

Table 5.4 Correlation between Ca^{2+} fluxes and several parameters

	Mn Content	Mn-tolerance	SPAD	Relative.SPAD	Fresh Weight (Shoot)	Fresh Weight (Root)	Dry Weight (Shoot)	Dry Weight (Root)
P-Flux	0.2349	-0.3786	-0.2365	-0.2839	-0.4331	-0.2646	-0.2560	-0.0324
S-Flux	0.5202	-0.6347	-0.3888	-0.5794	-0.2897	0.0164	-0.2032	0.2692
Total Ca2+	0.3216	-0.6359	-0.3686	-0.4881	-0.2708	-0.1221	-0.1166	0.1189

* If r≥0.632, P<0.05

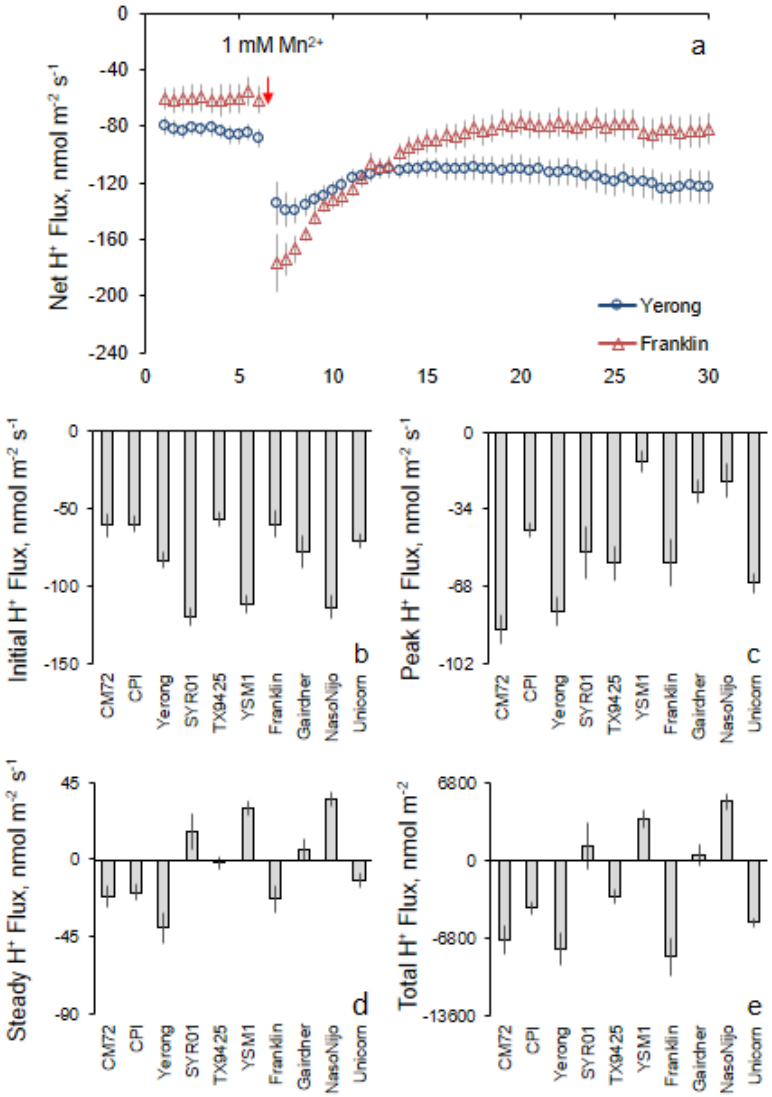

Fig. 5.5 (a) A typical example of transient change of H^+ flux upon the addition of 1 mM Mn^{2+} to barley root mature zone of Yerong (tolerant) and Franklin (sensitive). The arrow indicates commencing of the treatment. The magnitude of H^+ flux was determined as follows: (b) Initial H^+ flux; (c) Peak H^+ flux; (d) Steady H^+ flux; (e) Total H^+ flux. Data present as mean ± se (n = 6 – 10).

Table 5.5 Correlation between H^+ fluxes and several parameters

	Mn Content	Mn-tolerance	SPAD	Relative.SPAD	Fresh Weight (Shoot)	Fresh Weight (Root)	Dry Weight (Shoot)	Dry Weight (Root)
P-Flux	0.5515	-0.3124	-0.3764	-0.5067	-0.8198	-0.5436	-0.7108	-0.4542
S-Flux	0.4440	-0.2911	-0.3751	-0.4487	-0.6518	-0.5089	-0.5385	-0.3981
Total H+	-0.2039	-0.1271	-0.3470	-0.0771	-0.1327	-0.4348	-0.2316	-0.4217

* If r≥0.632, P<0.05

Significant H^+ efflux was measured from the mature zone. A similar trend was also observed in Yerong and Franklin in the response to 1 mM Mn (Fig. 5.5a). Net H^+ efflux of 57 to 120 nmol m^{-2} s^{-1} was measured under control (initial-state) conditions from barley roots (Fig. 5.5b). Genotypes showed different responses to

1 mM Mn treatment. Among them, tolerant genotypes CM72 and Yerong had stronger response than sensitive genotypes Gairdner and Naso Nijo, with almost 4-fold higher H^+ efflux (Fig. 5.5c). Most tolerant genotypes including Yerong, CM72 and CPI-71284-48 showed an ability to maintain a more negative net H^+ flux during steady state (Fig. 5.5d) while similar results were also showed in total H^+ flux (Fig. 5.5e). Interestingly, both peak H^+ flux and steady H^+ flux showed a significant correlation with shoot fresh weight (harvested after 10 days Mn treatment) (Table 5.5). It is likely that maintenance of H^+ pumping improves the ability of whole plant survival with better water retention.

5.3.3 Isotope tracer measurement

Tolerant genotypes CPI, Yerong and CM72 maintained high concentration of chlorophyll and contained less Mn in their leaves (Fig. 5.6a and 5.6b, respectively). There was no significant (at $P < 0.05$) difference in Mn uptake rate observed in root among those genotypes (Fig. 5.6c). However, three sensitive genotypes (Gairdner, Naso Nijo and Franklin) transported more Mn to the shoot compared with tolerant genotypes (Fig. 5.6d). There was a strong positive correlation between Mn content in shoots and shoot/root Mn concentration ratio with r = 0.95 (Table 5.6).

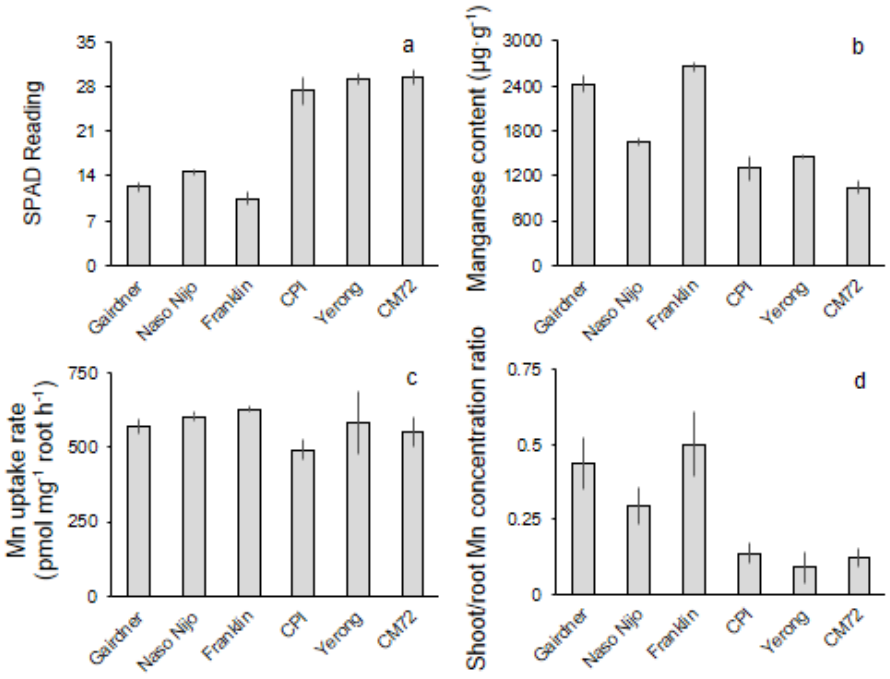

Fig. 5.6 Isotope tracer measurement and two physiological parameters of six barley genotypes under 1mM Mn^{2+} concentration treatment. (a) Chlorophyll content in SPAD reading; (b) Manganese content in shoot; (c) Mn uptake rate in root; (d) Shoot/root Mn concentration ratio. Data present as mean ± se (n = 3 - 5).

Table 5.6 Correlation coefficients between physiological and various isotopes parameters of 6 barley genotypes grown in 1 mM Mn^{2+} concentration

	Mn content in shoots	SPAD	Mn uptake rate
SPAD	-0.90		
Mn uptake rate	0.61	-0.64	
Shoot/root Mn concentration ratio	0.95	-0.97	0.60

* If $r \geq 0.81$, $P<0.05$

5.3.4 Pharmacology

To reveal the molecular identity of transporters mediating manganese-induced changes in root fluxes reported in Fig. 5.3 - 5.5, the effects of various channel blockers and metabolic inhibitors were studied using variety Franklin exposed to 1 mM Mn^{2+} stress.

All the inhibitors used had a significant impact on K^+ flux (Fig. 5.7). Initial K^+ uptake from mature zone of barley roots was strongly suppressed by TEA, DPI and vanadate. The strongest effect was observed for DPI, with initial flux turning negative (net efflux; Fig. 5.7a). Application of TEA and vanadate resulted in net zero K^+ flux both at initial (Fig. 5.7a) state and final (Fig. 5.7c) steady-state but induced a higher peak K^+ efflux than non-pretreated roots when applied Mn treatment (Fig. 5.7b). Effects of La^{3+} and Gd^{3+} were much smaller (Fig. 5.7b, c and d). In comparison, roots pretreated with DPI became more sensitive to 1 mM Mn^{2+}, achieving a highest K^+ efflux at almost 400 nmol m^{-2} s^{-1} (Fig. 5.7b). A significant K^+ leakage was also observed at steady-state, and consequently resulted in a substantial total K^+ loss (Fig. 5.7c and d).

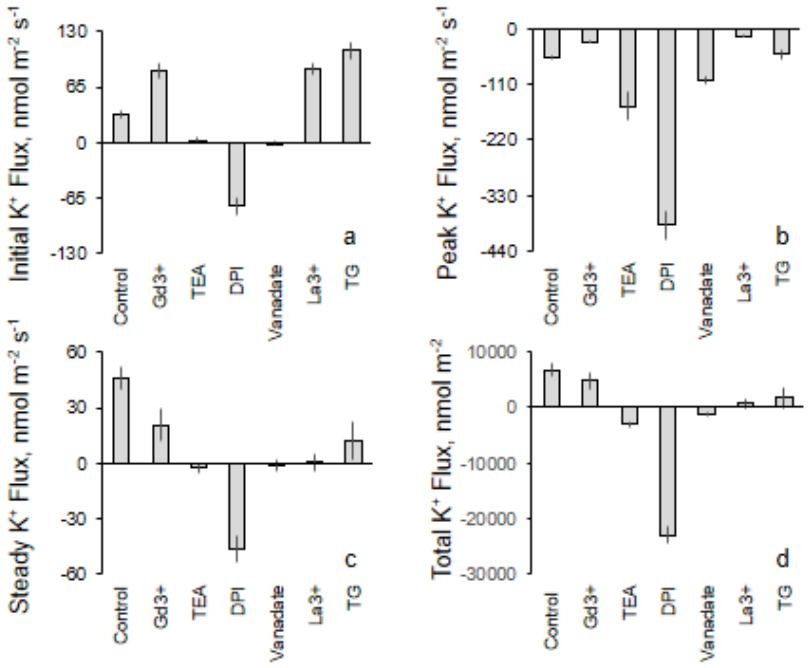

Fig. 5.7 Pharmacology of K^+ fluxes in mature zone of barley roots (Franklin) responses to 1 mM Mn^{2+}. The magnitude of K^+ flux was determined as follows: (a) Initial K^+ flux; (b) Peak K^+ flux; (c) Steady K^+ Flux; (d) Total K^+ flux. Data present as mean ± se (n = 6 – 10). Roots were pretreated with selected inhibitors for 1 h before Mn was added (still in the presence of inhibitor).

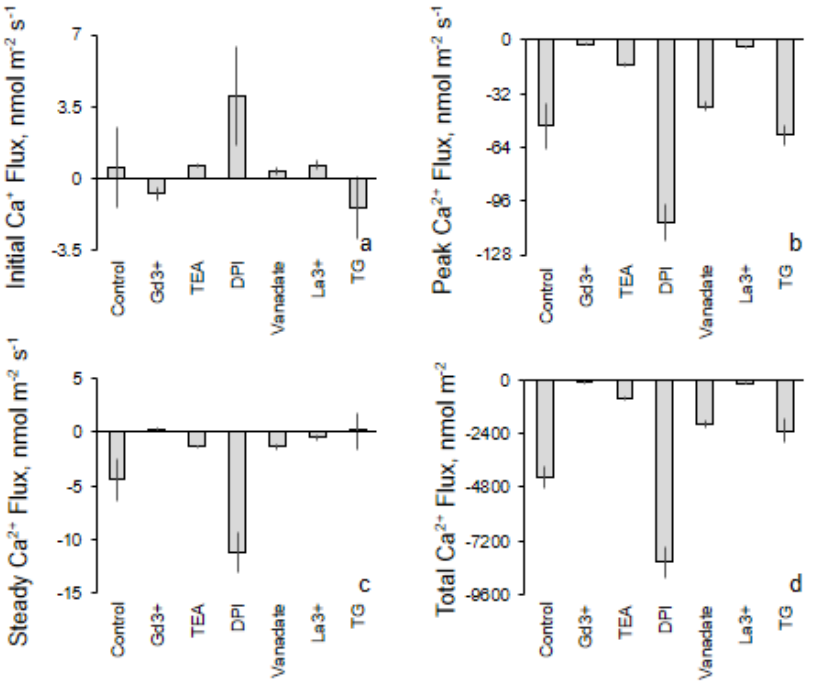

Fig. 5.8 Pharmacology of Ca^{2+} flux in mature zone of barley root (Franklin) responses to 1 mM Mn^{2+}. The magnitude of Ca^{2+} flux was determined as follows: (a) Initial Ca^{2+} flux; (b) Peak Ca^{2+} flux; (c) Steady Ca^{2+} Flux; (d) Total Ca^{2+} flux. Data present as mean ± se (n = 6 – 10). Roots were pretreated with selected inhibitors for 1 h before Mn was added (still in the presence of inhibitor).

None of the inhibitors substantially affected the initial Ca^{2+} flux after 1 h of incubation. However, DPI still slightly enhanced Ca^{2+} uptake in initial-state (Fig. 8a). A significant decrease of Ca^{2+} efflux was observed when roots were pretreated with TEA under Mn treatment, although TEA does not affect Ca^{2+} flux directly (Fig. 5.8b). At the same time, Gd^{3+} and La^{3+} significantly inhibited Ca^{2+} flux responses to Mn^{2+} treatment (Fig. 5.8b, c and d), with almost net zero Ca^{2+} flux at steady-state (Fig. 8c). The effect of TG was minor compared with control in peak flux (Fig. 5.8b), whereas it still caused almost 50% reduction of total magnitude of Ca^{2+} loss (Fig. 5.8d). Similar as effect on K^+ flux, root became more susceptible when pretreated by DPI, resulting in significant increase of Ca^{2+} efflux (Fig. 5.8b, c and d).

5.3.5 Mn ion induces transient membrane hyperpolarisation

Root's ability to maintain negative membrane potential (MP) is essential for providing a driving force for uptake of essential cations. As most membrane transporters or ion channels, particularly those for K^+, are voltage dependent (Ward *et al*. 2009), the ability to control cell MP could be critical in K^+ retention. The average MP in the mature zone of barley roots of variety Franklin was -120.7 ± 1.0 mV in the control condition. Application of 1 mM Mn^{2+} caused a significant hyperpolarization of about 30 mV stabilising at -149.9 ± 3.1 mV (Fig. 9). This result might explain significant K^+ uptake under excess Mn condition.

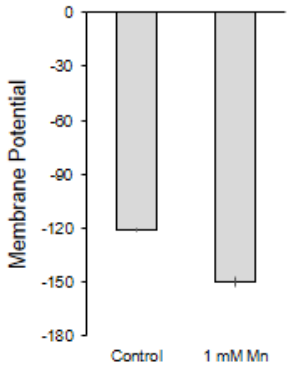

Fig. 5.9 Cell membrane potentials in the mature zone of roots of Franklin after 1 mM Mn^{2+} treatment in comparison with control. Data are means ± se (n = 8).

5.3.6 Mn decreases sensitivity to exogenous H_2O_2

We then investigated a possible role of Mn treatment in sensitizing/desensitizing root ion flux responses to ROS. Transient ion fluxes were measured from barley roots in response to 10 mM H_2O_2 after 5 d treatment under four different conditions (control, 1 mM Mn, hypoxia and hypoxia plus 1mM Mn). Addition of 10 mM H_2O_2 led to a gradual reduction of K^+ uptake, resulting in substantial K^+ efflux after 15 min (Fig. 5.10a). Roots pretreated with hypoxia became more susceptible and achieved a significant increase of the magnitude of K^+ flux in initial-state and steady-state (Fig. 5.10b and 5.10c). However, Mn-pretreated roots were observed an approximately 24% reduction of the magnitude of K^+ efflux when subjected to H_2O_2 compared with control plants ($P < 0.05$). Similarly, almost 40% H_2O_2-induced K^+ loss was prevented when roots were pretreated with Mn under hypoxia condition (Fig. 5.10d).

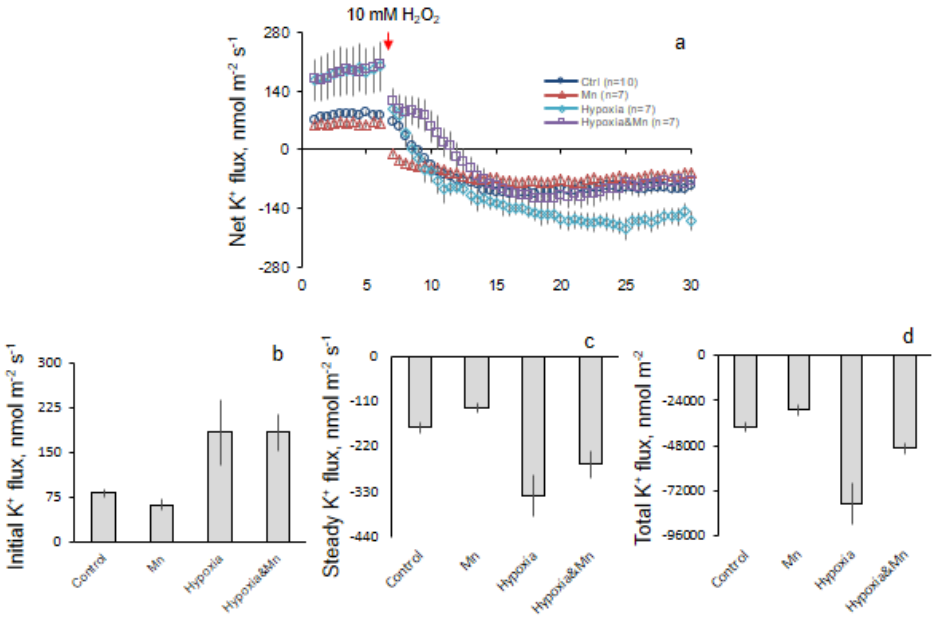

Fig. 5.10 (a) A typical example of transient change of K^+ fluxes upon the addition of 10 mM H_2O_2 to the mature zone of roots of Franklin. The arrow indicates commencing of the treatment. The magnitude of K^+ flux was determined as follows: (b) Initial K^+ flux; (c) Steady K^+ flux; (d) Total K^+ flux. Data present as mean ± se (n = 6 – 10). Roots were pretreated with four different treatments (control, 1 mM Mn^{2+}, hypoxia, and hypoxia plus 1 mM Mn^{2+}) for 5 d.

On the contrary to K^+, exogenous 10 mM H_2O_2 triggered a substantial Ca^{2+} uptake (Fig. 5.11a). In initial-state, a small Ca^{2+} efflux (7.6 ± 2.0 nmol m^{-2} s^{-1}) was measured in hypoxia-pretreated roots while others remained Ca^{2+} uptake (Fig. 5.11b). During the period of addition of 10 mM H_2O_2, there was no significant difference that was observed in peak Ca^{2+} flux (Fig. 5.11c). Consistent with results of K^+ flux, hypoxia enhanced the sensitivity to H_2O_2, and significantly elevated Ca^{2+} uptake. Addition of Mn ion efficiently alleviated the impact from hypoxia (Fig. 5.11d).

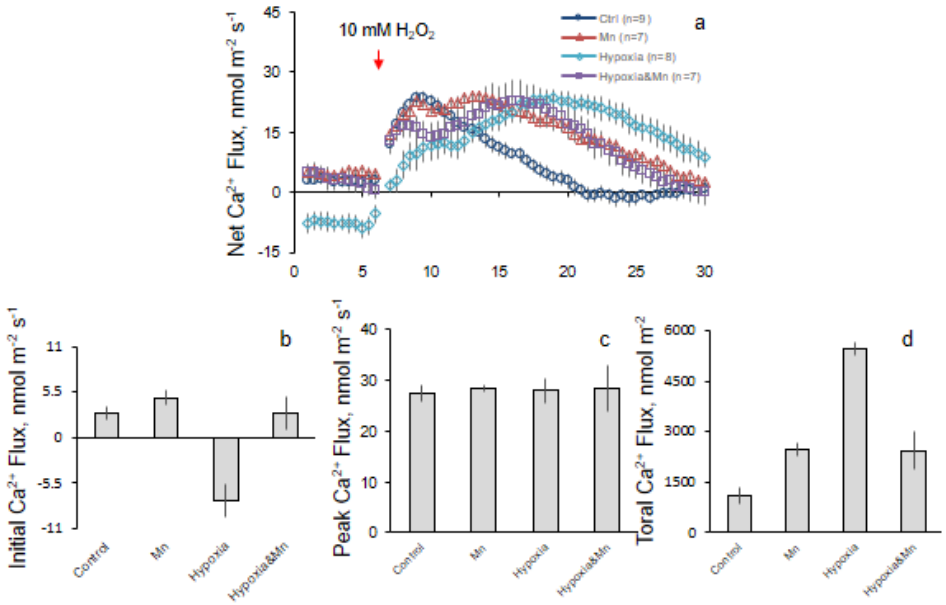

Fig. 5.11 (a) A typical example of transient change of Ca^{2+} fluxes upon the addition of 10 mM H_2O_2 to the mature zone of roots of Franklin. The arrow indicates commencing of the treatment. The magnitude of Ca^{2+} flux was determined as follows: (b) Initial Ca^{2+} flux; (c) Peak Ca^{2+} flux; (d) Total Ca^{2+} flux. Data present as mean ± se (n = 6 – 10). Roots were pretreated with four different treatments (control, 1 mM Mn^{2+}, hypoxia, and hypoxia plus 1 mM Mn^{2+}) for 5 d.

5.3.7 Mn up-regulates expression of HAK gene

We then investigated if Mn treatment may regulate root K^+ homeostasis at transcriptional level, and whether the resultant Mn-induced increase in K^+ uptake may be a result of upregulation of some K^+ uptake system. Two candidate genes (*MLOC_10758.2* and *MLOC_16004.1*, respectively) code *HvHAK5* and *HvHAK4* respectively in the barley genome database (International Barley Genome Sequencing Consortium 2012), which are located on chromosome 3H (see *Chapter*

IV). Both of them were suggested to be putative high-affinity potassium transporters, which were identified in *Arabidopsis* (Grabov 2007). The coding DNA sequence (CDS) can be found in Table S1. After transferred 3-day-old seedlings into BSM containing 1 mM Mn^{2+} for 2 h, both *HvHAK4* and *HvHAK5* slightly increased in the root tissue (relative to transcript level of *HvActin*). When the treatment period was increased to 24 h, there was no significant difference for transcript level of *HvHAK4* gene. For *HvHAK5*, however, the transcript level increased more than 150 folds (Table 5.7) compared with only 1.15-fold increase for 2 h treatment.

Table 5.7 Transcript level of *HvHAK4* and *HvHAK5* gene expression in root in Franklin (3-day-old seedlings grown under 1 mM Mn^{2+} for 2h and 24 h, respectively). Data present as mean ± se (n=9).

	2h	24h
HvHAK4	1.19 ± 0.34	1.26 ± 0.55
HvHAK5	1.15 ± 0.20	176.48 ± 14.76

5.4 Discussion

5.4.1 The difference in Mn tolerance in barley is not related to the rate of Mn acquisition by roots

Barley varieties used in this study possessed differential sensitivity to Mn (as evident from chlorophyll data; Fig. 5.6a). This difference was ultimately related to the cultivar's ability to reduce accumulation of Mn in the shoot (Fig 6b). At the same time, studies involving ^{54}Mn radiotracers revealed no significant difference in the rate of Mn uptake in root by cultivars (Fig. 5.6c). These results are in a good agreement with previous reports attributing the genotypic difference in Mn tolerance to ionic relations in shoots (Huang *et al.* 2015). However, the fact that tolerant barley cultivars Yerong had the same rate of Mn uptake as sensitive varieties Franklin but accumulated almost 50% less Mn in the shoot suggests that root ability to sequester Mn (presumably, in vacuoles; away from metabolically

active sites) is an important component of the Mn tolerance mechanism. Until now, Mn^{2+} tolerance has been causally related to the variation in Mn distribution and speciation within leaves (Mendoza-Cózatl *et al*. 2011; Blamey *et al*. 2015). Amongst two key mechanisms, Mn^{2+} sequestration in leaf vacuoles (essential for maintaining low cytosolic Mn^{2+} concentrations) and oxidation of Mn^{2+} to Mn^{3+} in trichomes (for reducing Mn content in the apoplast) were named (Blamey *et al*. 2015). Here we add an additional mechanism to this list, namely the higher ability of tolerant genotypes to sequester Mn^{2+} in root tissues. Future studies involving fluorescent dyes and/or SIMS techniques are needed to investigate which tissue acts as a major Mn sink for this process.

As for the molecular identity of transport systems mediating Mn sequestration in root vacuoles, the most likely candidates are CAX exchangers. CAX exchangers belong to the multigene family of cation/H^+ exchangers (Shigaki *et al*. 2006; Martinoia *et al*. 2011; Pittman and Hirschi 2016) that are known to operate as high affinity Ca^{2+}/H^+ exchangers involved in restoring cytosolic Ca^{2+} levels by sequestering it in vacuoles upon environmental stimulus (Bose *et al*. 2011). Typically, each plant species contains between four to fourteen CAX genes (Manohar *et al.* 2011; Pittman and Hirschi 2016). In the *Arabidopsis* genome, six CAX genes (*AtCAX1* to *AtCAX6*) have been identified (Manohar *et al*. 2011). However, some CAX isoforms were shown to also have affinity for other divalent cations, including manganese. Mei *et al*. (2009) reported an increased sensitivity in *cax4 Arabidopsis* loss-of-function mutant. Yeast cells expressing the N-terminal truncated CAX1 ORF were able to grow on high Mn^{2+} media (Shigaki *et al*. 2010), and tobacco plants overexpressing *CAX2* and *CAX4* genes showed increased Cd^{2+} and Mn^{2+} stress tolerance (Koren'kov *et al*. 2007). Interestingly, Mn-induced Ca^{2+} efflux flux activation was higher in Mn-tolerant varieties (Fig. 5.4c and 5.4e) and correlated positively with overall Mn tolerance (Table 5.4). Thus, it is plausible to suggest that higher Mn vacuolar sequestration ability in roots of tolerant barley genotypes is causally related to higher CAX activity. This suggestion is in line with the recent findings showing that the loss of CAX11 function results in waterlogging-sensitive phenotype in *Arabidopsis* (Wang *et al*. 2016). Future experiments should reveal whether this mechanism is conferred at transcriptional or post-translational levels, or both.

5.4.2 Mn treatment stimulates K^+ uptake and offset detrimental effects of hypoxia

Contrary to other stresses such as salinity (Chen *et al.* 2005; Wu *et al.* 2015) or hypoxia (Zeng *et al.* 2013), Mn treatment led to increased net K^+ uptake in the root (Fig. 5.3e) but not in the shoot mesophyll (Fig. 5.2a) cells. This may be indicative of two different mechanisms operating in these tissues that regulate K^+ homeostasis and, ultimately, cell fate (Shabala *et al*. 2016).

Maintaining intracellular K^+ homeostasis is critical for multiple cellular processes, including turgor maintenance, osmoregulation, leaf and stomata movements, tropisms, enzyme activation, control of membrane polarization in xylem loading, charge balancing, cytoplasmic pH regulation, protein and starch synthesis and energy conservation across membranes (Shabala, 2003; Dreyer and Uozum, 2011; Shabala and Pottosin, 2014). High cytosolic K^+ is also essential to prevent stress-induced programmed cell death (Shabala 2009; Demidchik 2014). In this context, a dose-dependent Mn-induced K^+ loss from shoots mesophyll cells (Fig. 5.2a) may be causally related to leaf chlorosis and ultimate death of mesophyll cells, as reported in the literature (Anschütz *et al*. 2014; Shabala and Pottosin 2014)

How can roots avoid K^+ loss? Two contributing mechanisms have been unveiled by this study. One of them is Mn-induced activation of H^+-ATPase, as evident from the stimulus-increase net H^+ efflux (Fig. 5.5d) and that result in a significant (about 30 mV; Fig. 5.9) membrane hyperpolarization, thus enhancing K^+ uptake in the root (Fig. 5.3e). Plant roots harbor a set of hyperpolarization-activated inward-rectifying K^+ channels such as AKT or KAT (Very and Sentenac 2003) that would operate in a low-affinity mode and mediate K^+ uptake under conditions in our experiments. The second mechanism is a massive increase in *HAK5* transcript levels (Tab. 5.7) that mediate high-affinity K^+ uptake (Grabov 2007; Alemán *et al*. 2011) and thus can supply sufficient amounts of K^+ not only for root consumption but also to be sent to the shoot. This mechanism may become critical for conditions when plant ATP pool is limited (as under hypoxia) and Mn-activated stimulation of H^+-ATPase become not feasible. It has been reported that root K^+ uptake is notably reduced upon oxygen depletion (Elzenga and van Veen 2010). Mn-induced increase in *HAK5* transcript levels may overcome this limitation.

5.4.3 Mn effects of root ionic homeostasis are linked with ROS signalling and mediated by the plasma membrane NADPH oxidase

Another interesting observation from this work was the beneficial effect of Mn application on root K^+ retention under hypoxic conditions (Fig. 5.10) and the observed desensitizing of roots to ROS (H_2O_2) stress (Fig. 5.10d). A broad range of ROS- activated channels mediate movement of Ca^{2+} and K^+ across cellular membranes (Demidchik and Maathuis 2007; Demidchik *et al.* 2003, 2010; Shabala and Pottosin 2014). Our pharmacological data shown in Fig. 5.7 suggests that both K^+ selective (blocked by TEA) and non-selective cations (blocked by Gd^{3+} and La^{3+}) channels mediate Mn-induced ion transport across the root membrane.

It was also shown that the plasma membrane-based NADPH oxidase plays an essential role as a positive regulator of ROS production and amplifies stress-induced Ca^{2+} (Lecourieux *et al.*, 2002; Demidchik and Maathis 2007) and K^+ (Shabala *et al.* 2015; Demidchik and Shabala 2017) signalling. Here we show that inhibition of NADPH oxidase by DPI has caused a biggest perturbation to ion fluxes (Fig. 5.7 and 5.8) implicating NADPH involvement in Mn responses.

Regardless of these details our findings of desensitising ROS sensitivity reported here suggest that the presence of elevated concentration of Mn in flooded soils caused by the shift in redox potential (Zeng *et al.* 2013) may play some beneficial role onto root ionic homeostasis and root operation under flooding stress conditions. In this context, these results are reminiscent of the recent reports showing that root conditioning with hypoxia can increase Al and acid stress tolerance in barley by mitigating activation of K^+ efflux channels by ROS (Ma *et al*. 2016). This calls for more efforts to be put into the study of cross-tolerance mechanisms and interactions between various constraints imposed by the same environmental stress onto the plant performance under the field conditions.

In conclusion, via the electrophysiological investigation of barley response to Mn toxicity, elevated concentration of Mn may be beneficial to plant survival under hypoxia condition. In this context, Mn may play a substantial role in conferring a better K^+ retention in root to waterlogging stress tolerance. Multiple mechanisms involve in this signal pathway by activating numerous K^+, non-selective and divalent cation channel or specific transporters. However, the specific mechanism

of desensitising of ROS sensitivity in hypoxic-treated roots by Mn remains to be answered in future studies.

Table S1 CDS of *HvHAK4 and HvHAK5*

>MLOC_16004.1 *HvHAK4*

atggacgccgaggccggcgccgtcgccgccgcccaggagcttccgtggaggcagcactacaggaatctactgctgctagcataccagagcttcgg
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gtg

>MLOC_10758.2 *HvHAK5*

atggccgagcctctgaagaccaacggcaatggagccgctgaaggggctctgcgtttgcgtcggtgaaggtgccgccgccgccgtcgccgccgag
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gaaacaaatgaaaggttcctcttccgatacgtggaaccaagagaatacagggtattccgatgtgtggtgcgctatggttacaacaataaagtagaag
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cagaatgccggtttggtgaagaaaataatagttgactacgcctacaatttcatgaggaagaacttcaggcaaccagagaagatcacatgtgtccctca
taacaggttgctgcgggtgggaatgacatatgagatc

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Chapter VI: General discussion and future research

6.1 General discussion

Plant waterlogging tolerance is a polygenic trait conferred by multiple mechanisms and affected by genetic background, physiological interactions in plants, and environmental impacts. Improving waterlogging tolerance of cereal crops is a key approach to secure the food supply in the future, in the light of a decline in yield of crops resulting from increasing issue of soil waterlogging. Due to the complex nature of waterlogging stress, the lack of a comprehensive understanding of underlying physiological and molecular mechanisms of waterlogging stress tolerance, and the lack of reliable and comprehensive screening methods impede the improvement in breeding programme in cereal crops (Zhou 2010). Most of the research effort on waterlogging tolerance has been focused on traits related to increased oxygen availability, such as preventing oxygen loss from non-meristematic root tissues or improving oxygen transport to, or storage in, the root (Jackson and Armstrong 1999; Setters and Waters 2003; Zhang *et al.* 2016). In addition to this, the tolerance to one or more ion toxicities can be also an essential trait to improve plant performance in waterlogged soils (Khabaz-Saberi *et al*. 2006).

There was a large number of existing barley genotypes which showed significant differences in waterlogging stress tolerance to develop of a rapid screening method of manganese toxicity tolerance using selection with a number of physiological parameters. In general, variation in plant biomass is used as one of the major criteria in screening for stress tolerance (Moroni *et al*. 1991; Scott *et al*. 1998; Khabaz-Saberi *et al*. 2010). However, relative root and shoot dry weight appear to be unreliable for screening for Mn tolerance, most likely due to relatively short timeframe of the stress (Fig. 3.6 and Table 3.1 in *Chapter III*). We concluded that the SPAD measurements of chlorophyll content and visual symptom scores on plants treated with 1 mM Mn^{2+} for 10 days can be used as reliable criteria in preliminary selection for tolerance to manganese toxicity (Huang *et al.* 2015). Until now, Mn^{2+}-use efficiency was the major aspect in most of the previous studies (Pallotta *et al.* 2000; McDonald *et al.* 2001; Hebbern *et al.* 2005). In the studies on waterlogging stress, several QTL controlling different traits have been reported in barley (Li *et al.* 2008; Zhou 2011; Zhang *et al.* 2016). However, no QTL has been

reported for the tolerance to Mn^{2+} toxicity. In our work (see *Chapter IV*), the novel QTL conferring manganese toxicity tolerance were identified. Four significant QTLs for plant survival on chromosome 1H, 3H, 4H and 6H, respectively, and two for the severity of leaf chlorosis on chromosome 3H and 6H under Mn^{2+} stress in the early growth stage were identified in the Yerong/Franklin population (Tab. 4.1 in *Chapter IV*). One minor QTL controlling plant survival under manganese stress (*QSur.yf.4H*) was located at a similar position as the major QTL for waterlogging tolerance based on the final plant survival score (Zhou 2011). Only one major QTL for plant survival was identified on chromosome 3H in the DH population of TX9425/Naso Nijo. Among those parent genotypes, TX9425 apparently had a worse performance under Mn treatment than Yerong. These results suggested that other minor QTL contributed to a better tolerance in Yerong compared with TX9425. It should be emphasised again that the Mn tolerance trait has never been targeted in the breeding programs aimed to improve plant performance under waterlogged conditions and, therefore, represents an untapped resource for plant breeders.

The present work (see *Chapter V*) has confirmed significant contribution and complexity of interaction to waterlogging stress tolerance (Fig. 5.10 and 5.11 in *Chapter V*). Among twenty barley genotypes selected in *Chapter III*, two main strategies of tolerance mechanisms (exclusion vs. internal tolerance) were found under Mn treatment. Exclusion mechanisms prevent Mn^{2+} from entering the cytosol and minimize harmful effects in the apoplast. Integrated with ^{54}Mn tracer measurements (Fig. 5.6 in *Chapter V*), tolerant barley cultivars had the same rate of Mn uptake as sensitive varieties but accumulated less Mn in the shoot (Fig. 3.7 in *Chapter III*), suggesting that the ability of roots to sequester Mn might be a dominant mechanism. Tissue tolerance mechanisms allow plants to take up and accumulate Mn^{2+} due to complexation, detoxification and compartmentalisation of Mn^{2+} within the plant. Until now, Mn^{2+} tolerance has been causally related to the variation in Mn distribution and speciation within leaves (Mendoza-Cózatl *et al*. 2011; Blamey *et al*. 2015). Mn^{2+} sequestration in leaf vacuoles and oxidation of Mn^{2+} to Mn^{3+} in trichomes are essential for maintaining low cytosolic Mn concentrations in the apoplast (Blamey *et al*. 2015). Several high-affinity Mn^{2+} transporters have also been reported in barley (Pedas *et al.* 2005), rice (Sasaki *et al.*

2012) and *Arabidopsis* (Cailliatte *et al.* 2010), and knockout or knockdown of the gene associated with these transporters resulted in decreased Mn^{2+} uptake (Sasaki *et al.* 2012), suggesting that Mn transporters may play an essential role in accumulation, long-distance transportation or cytosolic detoxification, such as CDF/MTP family, which efflux metals including Mn^{2+} out of the cytoplasm or into subcellular compartments (Gustin *et al.* 2011)

The present thesis has also advanced our understanding of the signal pathway

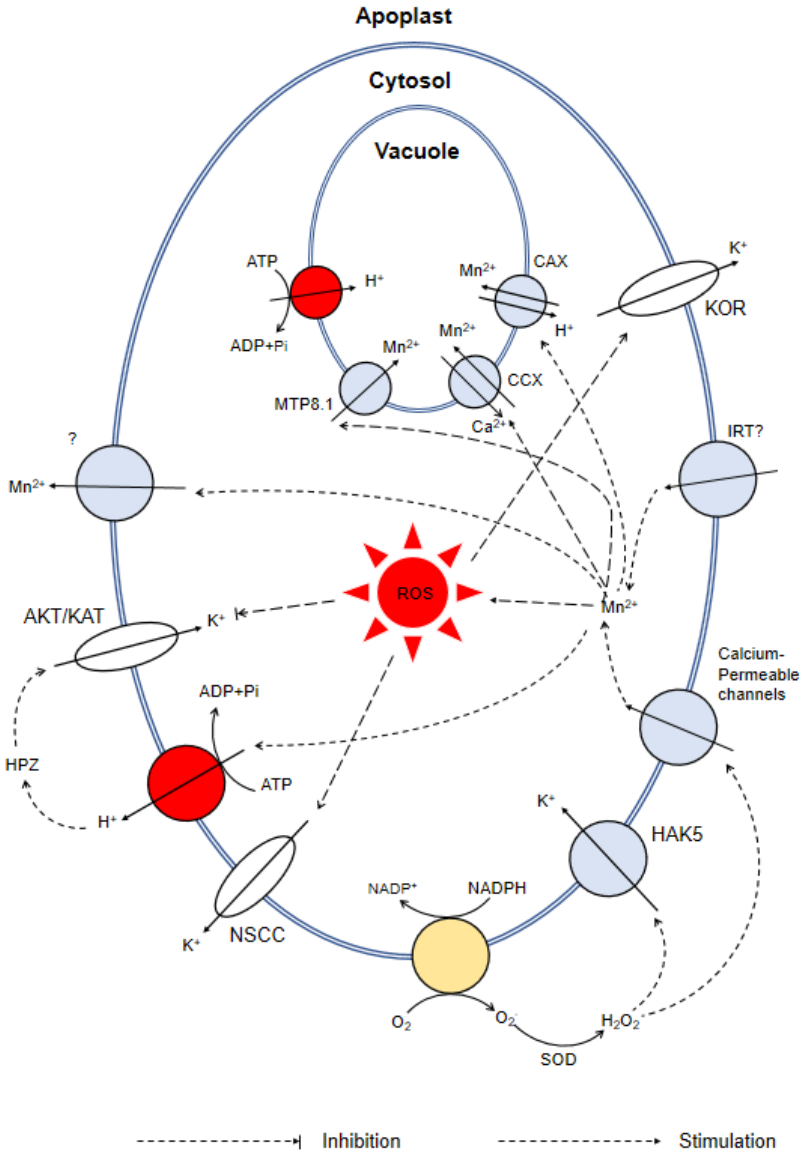

Fig. 6.1 Model depicting molecular mechanisms underlying regulation of Mn^{2+} transport in root.

under manganese toxicity (see *Chapter V*). Based on our reported results, a model summarises key mechanisms involved in control of ion transport under manganese

toxicity stress in barley roots (Fig. 6.1). K^+ is an activator of numerous metabolic enzymes (Dreyer and Uozumi 2011), including those for chlorophyll biosynthesis and photosynthetic CO_2 assimilation. Contrary to other stresses such as salinity (Cuin *et al.* 2011; Wu *et al.* 2015) or hypoxia (Zeng *et al.* 2013), Mn treatment can trigger a significant increase of net K^+ uptake in the root rather than in the shoot mesophyll cells (Fig. 5.3 and 5.4 in *Chapter V*), achieved by two major mechanisms. The first mechanism is that Mn-induced activation of H^+-ATPase increases net H^+ efflux when plants subject to Mn treatment. This causes the hyperpolarisation of plasma membrane, and then massive K^+ can be transported into cells through a set of hyperpolarisation-activated inward-rectifying K^+ channels such as AKT or KAT (Very and Sentenac 2003) (Fig. 6.1). The second mechanism was a significant upregulation in *HAK5* transcript levels (Tab. 5.7 in *Chapter V*) which could mediate high-affinity K^+ uptake (Boscari *et al.* 2009). This gene is encoded by MLOC_10758.2 which is located on chromosome 3H (QTL identified in Chapter IV). Therefore, once depolarisation of plasma membrane occurs due to lack of oxygen (Teakle *et al*. 2010; Zeng *et al*. 2014), Mn-induced increase in *HAK5* transcript levels may be responsible for maintaining K^+ uptake. Another observation also shows evidence of this Mn-induced K^+ retention under hypoxia condition (Fig. 10 in Chapter V) by desensitising of roots to ROS stress.

Further pharmacological investigation implies that plasma membrane-based NADPH oxidase involves in positive regulation of ROS production and other ionic signalling (Demidchik and Maathis 2007; Shabala *et al.* 2015; Demidchik and Shabala 2017) (Fig. 6.1). *AtHAK5* gene expression is dependent on the ROS production (Shin and Schachtman 2004; Jung *et al*. 2009). A member of the type III peroxidase family, RCI3, enhanced endogenous H_2O_2 level and up-regulated expression of *AtHAK5* (Kim *et al.* 2010). Also, our pharmacological data (Fig. 5.7 and Fig. 5.8 in *Chapter V*) suggests that both K^+ selective (blocked by TEA) and non-selective cations (blocked by Gd^{3+} and La^{3+}) channels mediate Mn-induced ion transport across the root membrane, and a group of cation channel including DACC (voltage dependent), HACC (ROS and voltage dependent) and NSCC (voltage dependent) also involve in Mn transport through PM, and CAX/CCX exchangers are likely to be responsible for Mn uptake and detoxification in cytosol (Mei *et al.*, 2007; Morris *et al.* 2008; McAinsh and Pittman, 2009).

Taken together, the result of this work confirmed several known mechanisms under manganese toxicity such as sequestration or detoxification of Mn in root, and discovered numerous unknown (or unutilised) traits conferring manganese toxicity tolerance including three candidate genes (*HAK*, *KAT* and *HMA*) from QTL findings, and improvement of K^+ retention, which contributed to waterlogging stress tolerance in barley. This knowledge and technological development have opened a new avenue for plant breeders in improving waterlogging tolerance in barley and minimising waterlogging-induced yield losses.

6.2 Future research

Among the study of barley genotypes, significant breakthroughs have been made on the mechanisms of manganese toxicity which are potentially contributing to a better waterlogging-tolerance. Nonetheless, several questions remain unknown in our knowledge:

1. One major novel QTL conferring manganese toxicity tolerance was identified in this study. It was confirmed that candidate gene *HvHAK5* plays an essential role in Mn-induced K^+ retention, consequently resulting in a better tolerance to hypoxia. However, due to the limited resolution of QTL mapping, the available markers might not pledge adequate reliability in tagging genes underlying these QTL. In this case, fine mapping a QTL can be critical to investigate and target this K^+ retention trait.
2. Two key potential mechanisms have been introduced in this thesis as compartment for Mn^{2+} sequestration (leaf vacuoles and trichomes in the shoots). Fluorescent dyes and/or secondary ion mass spectroscopy (SIMS) techniques are needed to investigate the specific tissue which acts as a major Mn sink in the cellular detoxification.
3. In this work, a tentative description of signalling pathways involved in Mn^{2+} transport through plasma membrane and vacuole has been revealed. However, the dynamic change of Mn concentration while interacting with other ions still remains numerous unknown. Carrying out one liquid ionic exchanger (LIX) specific to Mn ion may potentially solve this question.
4. Finally, a part of signalling pathways responding to manganese stress at the intracellular level has been described in root. However, Mn toxicity seems

to be more toxic in shoot rather than in root. Integrated with ^{54}Mn tracer measurements, more attention has to be paid to the long-distance transportation from root to shoot.

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